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The Digestibility of Polymerized Oils

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Introduction

Edible polymerized oils have, for many years, been used in small amounts in the food industry. In view of the recent increase in the use of these oils in foods (1, 2, 3), it was thought of interest to study their chemical and biological properties.

By suitable polymerization, unsaturated glycerides usually lose their characteristic odor and taste. As polymerization progresses, the viscosity of the oil increases, and the amount of unsaturation is decreased as measured by the decrease in iodine value. The emulsifying qualities of the oil also change characteristically as the molecular weight is increased. According to Bradley (4), the main structural changes that unsaturated glycerides undergo upon polymerization seem to involve their double bonds.

More recently, Brocklesby (5) has suggested that, during the polymerization of unsaturated glycerides, the most likely course is for two double bonds to react to form an unstable 4-carbon ring which breaks open and rearranges as follows:

$$\begin{array}{ccccc} R\cdot CH{=}CH\cdot R' & & R\cdot CH{-}CH\cdot R' & & R\cdot CH_2CH\cdot R' \\ + & \longrightarrow & | \qquad\quad | & \longrightarrow & | \\ R\cdot CH{=}CH\cdot R' & & R\cdot CH{-}CH\cdot R' & & R\cdot C{=}CH\cdot R' \end{array}$$

This type of polymerization, according to Brocklesby, might take place between two unsaturated fatty acids both attached to the same glyceride molecule (intramolecular) or between fatty acids of different glyceride molecules (intermolecular). Our results suggest that this reaction is more likely to occur between fatty acids of different glycerides than within the same glyceride. Furthermore, Brocklesby's theory for intramolecular ploymerization involves the elimination of two double bonds between adjacent fatty acid molecules within the same triglyceride, thereby forming a 4-carbon ring. Since this ring, as visualized by Brocklesby, is unstable, a shift in the position of one fatty acid chain with respect to the other takes place, resulting in the reformation of one of the two double bonds involved in the ring formation. The molecular structure of triglycerides makes such a change unlikely, as this undoubtedly would tend to rupture the glyceride molecule.

A considerable amount of research has been done on the polymerization of the methyl esters of unsaturated fatty acids (7, 8) and glycerides (4, 5, 6). The literature appears to contain few references to the biological effect of thermal treatment of edible oils (9).

To investigate this problem, a typical unsaturated oil was selected, namely, sardine oil (California pilchard, *Sardinops caerulea*). This oil is used, to a large extent, in animal nutrition as a carrier of vitamin A and D oils and is a natural constituent of fish meal, which has an extensive use in animal nutrition.

Experimental

A sample of refined destearinated sardine oil was subjected to polymerization by heating it in a glass vessel at 250°C. in a nitrogen atmosphere. By taking samples of the oil at regular intervals, and checking its iodine value and refractive index, the degree of polymerization was followed. Three samples of polymerized oil were produced having iodine values (Hanus) of approximately 160, 140, and 120, respectively. These samples showed only a moderate increase in free fatty acid over the original oil. They were, however, all subsequently alkali-refined, and this brought the free fatty acid content of all of them down below 0.5%. The samples were stored under nitrogen in a refrigerator until ready for use. The sardine oil, the samples of polymerized sardine oil produced therefrom, and a sample of commercially available edible polymerized herring oil, were subjected to a detailed analysis as shown in Table I.

To determine the effect polymerization has upon the biological value of sardine oil, a series of animal experiments were undertaken. It was decided to use rats as the experimental animal and to determine the coefficient of digestibility of the polymerized oils by the fat balance method. The details of the experiments were as follows:

Mature albino rats of the Sprague-Dawley strain weighing approximately 275 g. were divided into groups of 10 each, balanced according to sex and weight, and placed in individual metabolism cages so adapted that the urine and feces could be collected separately. The experiment was divided into 3 feeding periods, a preliminary depletion period of 4 days, followed by a test period of 7 days, and a final 3 day after period. During the depletion period all of the rats received a fat free basal diet *ad libitum*. During the 7 day test period the rats received the basal diet supplemented with 5% of the various oils under study. During this test period an accurate account was kept of the food intake, the various groups being restricted to 15 g. of diet/day/rat. During the after period the rats were again given the fat free basal diet *ad libitum*.

TABLE I

Effect of Polymerization on Physical Constants of Samples Tested

Sample	Iodine no. Hanus	Per cent unsaponifiable fraction	Refractive index 27°C. N_D	Per cent free fatty acid	Molecular weight[a]	
					Whole oil	Fatty acids[b]
Control. Sardine oil	177.7	1.61	1.4800	0.19	828	286
Polymerized sardine oil first fraction	155.5	2.09	1.4830	0.15	942	306
Polymerized sardine oil second fraction	138.1	2.45	1.4843	0.30	1062	319
Polymerized sardine oil third fraction	124.1	2.39	1.4858	0.50	1151	336
Polymerized herring oil commercial sample	106.4	1.61	1.4755	0.20	962	301

[a] Cryoscopic method, using benzene as a solvent.

[b] The oils were saponified, the unsaponifiable fractions were removed, and the molecular weight determinations were run on the free fatty acids resulting from acidulation of the soaps. A correction was made for dimerization.

The composition of the basal ration, and the rations fed during the test period are shown in Table II. It was decided to feed the oils at a level of 5% in the ration; this, it was thought, would compare more closely to the fat content of the average rat ration than the higher levels used by other workers in fat digestibility studies (10, 11, 12).

During the test period and after period, the feces were collected daily and stored as composite group samples in a measured amount of aqueous 0.5% formaldehyde solu-

TABLE II

Composition of Diets

Ingredient	Per cent: Fat-free basal diet	Per cent: Groups 2 through 6
Vitamin test casein G.B.I.	30.0	30.0
Cystine, C. P.	0.3	0.3
U.S.P. salt mixture No. 2	4.5	4.5
Rice bran concentrate (NOPCO)[a]	8.0	8.0
Ruffex, Fisher	5.0	5.0
Fat	—	5.0
Sucrose	52.2	47.2
2-Methyl-1,4-naphthoquinone	[b]	[b]

[a] Added with rice bran concentrate/100 g. of diet: Riboflavin 1.0 mg., Calcium pantothenate 2.5 mg., Chlorine chloride 50.0 mg.

[b] Added to all diets at a level of 5 γ/g. of diet.

tion. The animals appeared to be in good health and no deficiency symptoms were noticed. The inclusion of Ruffex in the diet eliminated the greasy consistency of the feces observed in an earlier experiment, thus minimizing any mechanical losses during their collection. Samples of urine from the various groups of animals were tested for pH, and the ketone bodies were determined using the method of Greenberg *et al.* (13). The results of these urine tests showed no significant trends. At the end of the experiment, the total amount of fat consumed by the individual groups was noted, and a fat determination on aliquots of the feces of the various groups was carried out using the Saxon method (14).

To obtain some information as to the nature of the unabsorbed fat, the balance of the fecal samples were used for a determination of the average molecular weights of the fatty acids. The fat contained in the feces consists to some extent of fatty acids, partially free and partially in the form of soaps, besides fatty acids in ester form. To obtain a sample of fatty acid for molecular weight determinations which would include fat in all the above-mentioned forms, but exclude sterols and any oxidized fats, the following analytical procedure was used.

TABLE III

Analytical Results of Feeding Experiment

Type of fat fed	Group	No. of rats	Total fat consumed	Total fat recovered in feces	Unabsorbed fat recovered in feces[a]	Coefficient of digestibility[b]	Molecular weight of fecal fatty acids[c]	Molecular weight of fatty acids from unabsorbed oil[d]
			g.	g.	g.			
None (Fat-free basal)	1	10	0.00	3.34	—		257	
Control sardine oil	2	10	49.67	4.19	0.85	98.3	280	374
Polymerized herring oil	3	10	49.75	5.87	2.53	94.9	399	587
Polymerized sardine oil, first fraction	4	10	49.68	5.33	1.99	96.0	345	492
Polymerized sardine oil, second fraction	5	10	49.73	8.56	5.22	89.5	413	513
Polymerized sardine oil, third fraction	6	10	49.25	10.85	7.51	84.8	565	702

[a] The correction for metabolic fat was made by subtracting 3.34 g., the metabolic fat recovered in Group 1, from each of the other values.

[b] Coefficient of digestibility as used here is defined as that fraction of total ingested fat which is retained.

[c] Average molecular weight of total fecal fatty acids determined by the cryoscopic method using benzene as solvent and correcting for dimerization.

[d] Calculated from the molecular weights of total fecal fatty acids by correcting for the influence of metabolic fat.

The samples of feces were saponified, and sterols and other unsaponifiable matter removed from the saponificate by a thorough extraction with low-boiling petroleum ether. After acidulation the fatty acids were removed from the aqueous solution by repeated ethyl ether extractions. Following evaporation of the ethyl ether, the fatty acids were finally isolated by re-extracting the residue with low-boiling petroleum ether. Molecular weight determinations were then carried out on the fatty acid samples using the same method as applied to the original polymerized oils.

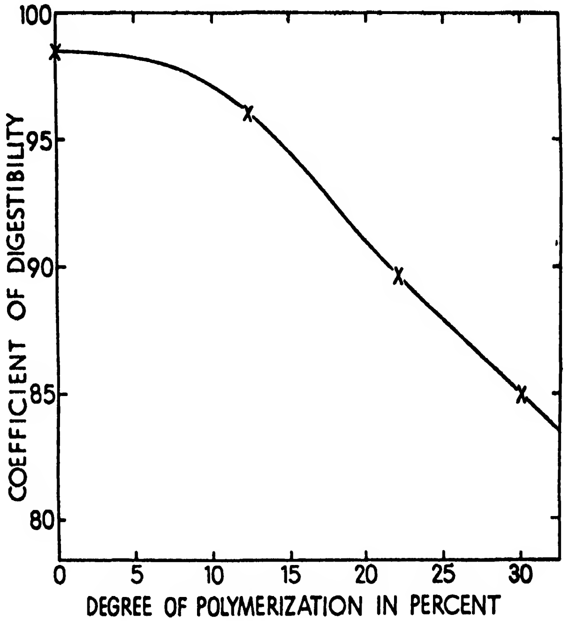

FIG. 1. Relation between degree of polymerization in per cent and the coefficient of digestibility of sardine oil. The degree of polymerization is defined as:

$$\frac{\begin{pmatrix}\text{Iodine number of} & & \text{Iodine number of} \\ \text{unpolymerized oil} & - & \text{polymerized oil}\end{pmatrix} \times 100.}{\text{Iodine number of unpolymerized oil}}$$

The results of the fat balance study, as well as the molecular weight determinations, are shown in Table III. Table III and Fig. 1 show, as expected, that the digestibility of polymerized oils decreases as the degree of polymerization is increased. Table III also shows that the molecular weights of the unabsorbed fat (as determined on the composite fatty acids of fecal fat) increase as fats of increasing degree of polymerization are fed.

Discussion and Results

The determination of molecular weights on the polymerized oils and their fatty acids does not reveal with any large degree of accuracy to what extent sardine oil may be polymerized intramolecularly or intermolecularly. The evidence does, however, speak in favor of a predominantly intermolecular polymerization under the conditions of our experiment. This conclusion is based on a comparison of the increase noted in molecular weight of the polymerized sardine oils with the increase in the molecular weight of their corresponding separated fatty acids. If it were purely intermolecular polymerization, the molecular weight of an unsaturated glyceride would double in case of complete dimeric polymerization, whereas the molecular weight of its fatty acids would only increase by about 20%. If, on the other hand, it were exclusively intramolecular polymerization, the molecular weight of such a glyceride would not increase at all, while that of its separated fatty acids would increase by about 50%.

The values in Table I show that the increase in molecular weight of sardine oil, due to polymerization, amounted to about 39%, whereas the increase in molecular weight of the corresponding separated fatty acids amounted to only 18%. These percentages favor intermolecular polymerization.

The healthy appearance of the animals at the end of these experiments, and the fact that no significant trends were discovered in the analysis of the urine from these rats with respect to pH or ketone bodies, would seem to indicate that the ingestion of a polymerized oil does not produce any visible metabolic disturbances in the rat. The increase noted in molecular weight of the fatty acids from the fecal fat to nearly double its value, although the degree of polymerization of the corresponding oil has only increased to about 30%, suggests that the polymerized portion of the oil is not absorbed to any large extent. Such an assumption would explain the lower digestibility that we have found for the polymerized oils.

The sample of polymerized herring oil, representing a commercially available oil which is being used in increasingly large amounts in the food industry, exhibited the same characteristics as the polymerized sardine oils. In addition to the lower digestibility found for polymerized sardine oil, preliminary experiments have indicated that feeding of these oils to rats on a vitamin K-free ration may produce vitamin

K deficiency symptoms in 7–10 days. These findings are being further investigated and will be reported on separately.

Acknowledgment

Our appreciation is expressed to Frank M. Campbell for his valuable assistance with the animal experiments.

Summary

The data presented indicate that during the polymerization of sardine oil, intermolecular dimers are largely formed. The coefficient of digestibility of the polymerized oils was compared with that of the unpolymerized oil and found to decrease with increasing degree of polymerization.

A determination of the molecular weights of the total fecal fatty acids (free as well as combined) indicated that the polymerized portion of the oils is not absorbed to any large extent, if at all.

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The Chemistry of the Living Bark of the Black Locust Tree in Relation to Frost Hardiness. I. Seasonal Variations in Protein Content [1,2]

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Introduction

The development of frost hardiness in plants is a phenomenon which has occupied the interest and attention of botanists and plant physiologists for years. Considerable advances have been made toward arriving at an understanding of the physiological phases of this phenomenon (1, 2), but the problem of the biochemical mechanism of the development of hardiness remains obscure. The application of the newer biochemical techniques, developed from the study of animal tissues, to the study of the problems of plant physiology, has led to considerable advances in the understanding of the processes of photosynthesis, metabolism, and salt accumulation in plants. With most plants of the north temperate zone, particularly the perennials, the development of frost hardiness is no less a part of their physiology than is photosynthesis or salt accumulation, but little has been done toward studying the problem from a biochemical point of view.

[1] Paper No. 2427. Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] The studies presented in this paper were initiated in 1942–43, while D. Siminovitch held a Royal Society of Canada Fellowship for study under the late Prof. R. A. Gortner at the University of Minnesota. The contents of this paper are condensed in part from a thesis presented by D. Siminovitch to the faculty of the Graduate School of the University of Minnesota in partial fulfilment of the requirements for the degree of Doctor of Philosophy, July, 1948, and in part from a continuation of the studies made under a grant from the Graduate School, University of Minnesota.

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The trees and shrubs of the forests in the north temperate zone exhibit remarkable adaptation to low temperatures since they survive winter temperatures of −20° to −50°C. or lower. That the mechanism of this adaptation is not a permanent feature of the tree or shrub throughout the year but is elaborated annually and alternately in response to the seasonal periodicity of climate is evident from the fact that these trees and shrubs will be killed even by −5°C. when frozen artificially in midsummer during their period of most active growth.

Chemical investigation of the nature of this frost resistance has been deterred be cause only recently has it been established that it is largely a property of the protoplasm of the living cell. Through the concerted efforts of a group working under G. W. Scarth, who had been occupied with the study of cell physiology over a period of many years, the problem has been considerably clarified. As a result of this work (3, 4, 5, 6, 7), it has become possible to establish directions for a biochemical approach to the problem. It has been shown that certain physical changes, microscopically observable and measurable, occur in the protoplasm and protoplasmic membrane of living cells when plants develop frost hardiness. These changes consist of an increase in the permeability of the protoplasmic membrane to water and other polar substances, an increase in non-solvent space in the cell and an increased capacity of the protoplasmic membrane to retain its plasticity after dehydration. The degree to which these living cells could withstand freezing is positively correlated with the extent of these physical changes, and a quick test of hardiness based on these protoplasmic changes has proven to be a valuable guide to studies on the real hardiness of plants. The intimate association of proteins and lipides with the cell, either as components of enzyme systems or of the structural organization within the cell, and the high surface activity of proteins and lipides which would indicate their participation in the structure of the surface of the cell suggest that changes in these constituents may be involved in the development of cold resistance.

This, the first in a series of studies dealing generally with the problem of the biochemistry of frost hardiness of tree cells, will report the results of an analysis of the seasonal variations which occur in the proteins of living bark cells when these cells undergo changes in their resistance to frost injury. Some observations on the variations which occur in the carbohydrates are included. Because some experiments which were conducted on the bark of stumps of trees from which the crowns had been removed in late winter, yielded information as to the relative importance of protein and carbohydrate in relation to frost hardiness, analytical results on this stump material have also been included in this report.

Experimental

Choice of Material for Study

The living bark of trees serves as excellent experimental material for an investigation of the biochemical changes associated with the development of frost hardiness.

In this cortex, the physical changes observable microscopically in the protoplasm during hardening and dehardening are greatest, occurring seasonally in autumn and spring, respectively. Because the living bark of the tree in winter is exposed to the greatest extremes of low temperature, it would be expected that the amplitude of change in hardiness, and, therefore, of physiological and chemical change, will be greatest in the cells of bark and should consequently be easiest to detect there.

The selection of a suitable species of potentially hardy tree was contingent upon the success with which extraction of proteins from the bark tissue could be made. Apple trees were first tried because of the availability of a large number of varieties possessing recognized different degrees of hardiness. However, very little water-soluble protein could be extracted from apple bark, as has been previously reported by Thomas (8) and others, or from the barks of the other species of trese which were examined.

The authors were directed to the black locust, *Robinia pseudo-acacia* L., by a publication of Jones, Gersdorff and Moeller (9), in which is described in detail the extraction and isolation of soluble proteins in considerable quantity from the bark tissue of this tree. These investigators used drastic extraction methods. The tissue was dried, ground thoroughly in a ball mill (both procedures being known to denature proteins), and then extracted with water or salt solutions. For the present studies, it was desirable that less drastic methods be used for maceration of the living bark and that the tissue used be in the fresh condition so that proteins could be extracted in the undenatured state.

Determination of Frost Hardiness

The availability of a reliable measure of hardiness was essential to the chemical investigation of the problem. The degree of hardiness of the bark cells must be known at any time that a chemical analysis is made if any relation between the hardiness and the chemistry of the bark is to be determined. The hardiness tests employed were based on studies of the properties of hardy and unhardy living cells (2), which showed that the degree of resistance of hardy cells to intercellular freezing can be estimated from the extent to which they can withstand dehydration, whether this is produced by plasmolysis in salt or sugar solutions or desiccation of the exposed cells in atmospheres of low relative humidities (10). Normally, when plant tissues are frozen, ice crystals grow in the intercellular spaces at the expense of water within the cells (5). The degree to which the cells are dehydrated during freezing is determined by the temperature to which they are exposed. Plasmolysis of the cells followed by deplasmolysis, or desiccation and subsequent rehydration of the cells, in effect reproduce the conditions observed when the tissues are frozen and thawed. The lowering of the freezing point, as calculated from the osmotic pressure of the solution which the cells can withstand without injury, is roughly indicative of the freezing temperature which they will survive and, therefore, of their hardiness.

The hardiness test on a bark sample was made as soon as possible after collection of the material. Thin, tangential sections were taken from the living bark and placed in a graded series of balanced salt solutions ($NaCl:CaCl_2::9:1$) of increasing concentration (1 M–5 M). Ten sections were plasmolyzed in each solution for 10 min. and then transferred to water. After an interval of 5 min., they were tested for survival by staining with neutral red solution (a few drops of a 1% solution in 25 cc. water).

If the cells absorbed and retained neutral red they were considered alive. An estimate was then made of the percentage of survival of the cells in each tissue section. The hardiness was recorded as the percentage of cells that remained alive after exposure to the various solutions. Since each tissue section contained over 100 cells, the estimate of survival after exposure to each of the solutions was based on at least 1000 cells.

It is to be noted that the seasonal course of frost hardening observed in the locust with this hardiness test corresponded very well with the seasonal course of frost hardening observed in apple trees by Hildreth as determined by actual freezing tests (11).

Analytical Procedures

1. Preparation of Material

The collection and treatment of material for these experiments were guided by the results of exploratory work done during 1942–43. Periodically through the fall, winter, spring, and summer of 1946–47, black locust trees 9–13 years old, from a single neighboring group, were cut near the base of the trunks and log sections 3 ft. in length and 3–4 in. in diam., and complete with outer and inner bark attached, were collected. The bark was promptly removed, the outer dead corky bark being peeled off with a knife and discarded, exposing the inner white living bark which was removed by holding the log horizontally against a rotating wire-brush wheel. Sufficient pressure was used to permit the wheel to brush lightly against the bark so as to avoid grinding the wood just underneath the bark. In this way the bark was shredded and removed uniformly over the length of the log. Large samples of shreds were collected for extraction of protein. Small samples were collected in moisture dishes for the determination of moisture and nitrogen fractionation. To obtain a representative sample of the bark, the small samples were usually taken in complete narrow 1 in. rings encircling the log at 1 ft. intervals. Sampling in this way was necessary, because the bark shreds were not readily mixed and the removal of a small representative sample from a large quantity of shreds was not possible.

2. Nitrogen Determinations on Small Fresh Bark Samples

Samples of fresh bark (equivalent to 4–5 g. dry matter) were thoroughly extracted with 150 cc. distilled water and filtered. The residue was then re-extracted with another 50 cc. water, filtered, and washed. The total filtrate and washings were combined, trichloroacetic acid added to form a 10% solution, and the precipitated proteins filtered off and washed with 10% trichloroacetic acid. Nitrogen determinations were made by the macro Kjeldahl method to secure values for the water insoluble nitrogen (insoluble protein in residue), the water soluble protein (trichloroacetic insoluble), and the water soluble non-protein nitrogen (soluble in 10% trichloroacetic acid).

3. Determinations of Soluble Protein in Large Fresh Bark Samples

Large samples of shredded bark (200–300 g.) were extracted with distilled water (10 g. water/g. bark) and filtered in the cold at 4°C. to yield clear, straw-yellow extracts. The extracts were saturated with $(NH_4)_2SO_4$, the heavy flocculum of protein collected by filtration and dialyzed against distilled water overnight at 4°C.

The sacs were then transferred to a buffer solution of total ionic strength of 0.20 consisting of a mixture of 0.19 ionic strength NaCl and 0.01 ionic strength phosphate at pH 7.2, and equilibrated against changes of this solution until free of sulfate.

The nitrogen content in these extracts was determined in an aliquot by a micro Kjeldahl method. The total nitrogen extracted was calculated from the volume of extract obtained in each case.

4. *Reducing and Non-reducing Sugar Determinations*

Determinations of reducing and non-reducing sugars were made on small air-dried samples according to the method described in "Cereal Laboratory Methods" published by the Am. Assoc. of Cereal Chemists (1947, p. 32).

5. *Moisture Determinations*

Moisture content of the bark tissue was determined by drying in a vacuum oven at 100°C. for 4 hr.

Results

The analytical findings are presented graphically in Figs. 1, 2, and 3. In each of these figures the results of the hardiness tests are shown as

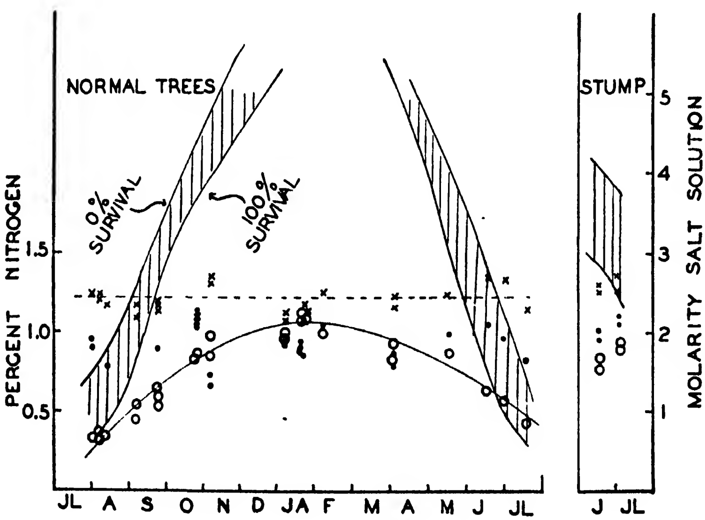

FIG. 1. Showing the annual variations in the hardiness and in the water-insoluble protein nitrogen, the water-soluble protein nitrogen, and the water-soluble non-protein nitrogen contents (per cent dry weight of bark tissue) of the living bark of normal trees of the black locust. Data obtained on bark of stumps of trees of the same species during the months of June and July are also shown. (Data taken from small samples.) (X) = non-soluble protein nitrogen. (O) = soluble protein nitrogen. (●) = soluble non-protein nitrogen.

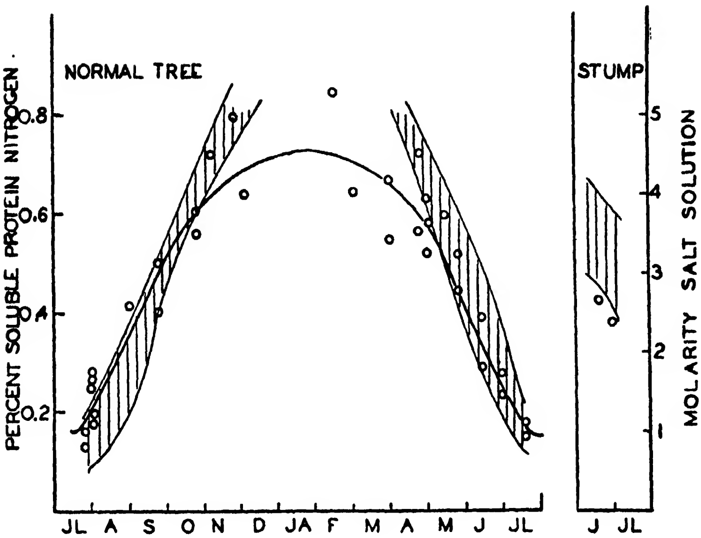

FIG. 2. Showing the changes in the hardiness and the water-soluble protein nitrogen (extracted from large samples of bark) in the living bark of the normal trees of the black locust during a year and in the bark of stumps of trees of the same species during the months of June and July.

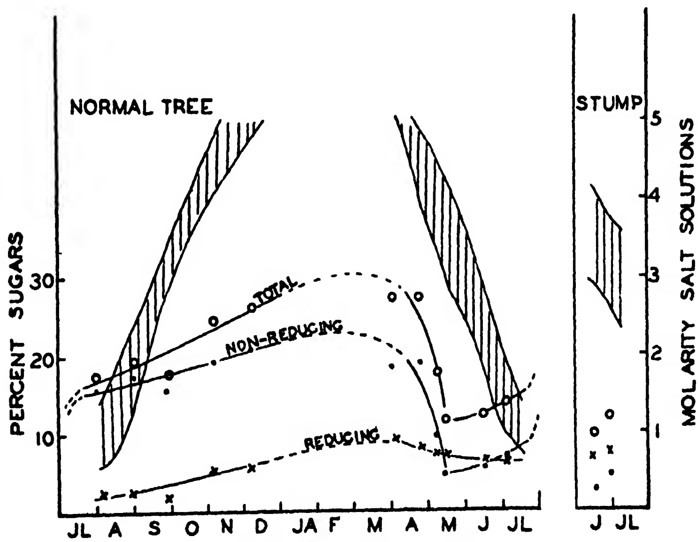

FIG. 3. Showing the variations which occur in the hardiness and in the soluble total, reducing, and non-reducing sugars in the living bark of normal trees of the black locust during a year and in the bark of stumps of trees of the same species during the months of June and July. (Data taken from small samples.)

a crosshatched area representing degrees of survival of cells between zero and complete survival after being subjected to the dehydrating action of the salt solutions of the concentrations indicated. Fig. 1 shows, in addition, the analytical values, in per cent of dry weight of tissue, for insoluble (protein) nitrogen, soluble protein nitrogen and soluble non-protein nitrogen obtained on the small samples of bark. Fig. 2 presents the data, calculated again as percentage nitrogen on the dry weight basis, for the soluble protein obtained by actual extraction from large samples of bark. Fig. 3 gives the analyses for sugars, total, reducing, and non-reducing, present in the bark at the time of collection of the logs from the field.

It must be recognized that some degree of variation will always occur between individual trees collected at the same time of year, that small variations in composition may occur between different logs from the same tree, and that, in the sampling technique employed, it has not been possible to obtain samples which are entirely free of outer bark and underlying woody material. Consequently, the extent of uncontrolled variation between the samples is rather large. Due to a limited number of trees available for these experiments, elimination of these uncontrolled variables by increasing the number of samples could not be attained. It is to be emphasized, therefore, that only variations which are relatively large between samples and which show a definite trend with respect to the measured hardiness and with the season of the year can be considered significant.

It may be noted also that the amount of nitrogen extracted as soluble protein from the large samples of bark is, in all cases, somewhat smaller than that extracted as soluble protein from small samples. This discrepancy undoubtedly arises from the circumstance that, while the procedure used in extracting all samples in each class was consistent, the degree of extraction of the large samples was not as exhaustive as that employed on the small samples.

Discussion

It is apparent from these analyses that, of the nitrogen fractions of the living bark cells of the locust tree, only that corresponding to the water-soluble proteins shows a direct and consistent correlation with the frost hardiness of these cells. The water-insoluble (protein) fraction appears, within a certain degree of variation, which includes that which normally exists between individual trees and which includes variations

in sampling accuracy, to remain constant through the year and is not related to hardiness variation. The water-soluble non-protein nitrogen, while showing a large degree of variation with time of year (a larger variation than is observed between samples from different trees which are collected at the same time) shows no correlation with the state of hardiness of the bark cells.

From the data presented in Fig. 3, it would appear that the soluble sugar content of bark cells also increases in late summer and autumn in a manner which is roughly proportioned to the development of hardiness. In the spring however, it is seen that the soluble carbohydrate content decreases precipitously with the initiation of sap flow (late April) and starts to increase again (in June) and these changes bear no close correlation with the dehardening of the cells. This is in marked contrast to the close correlation between soluble protein content of the cells and their state of hardiness observed through the same time period.

This absence of correlation of hardiness with soluble carbohydrate content of the cells and the contrasting close correlation with their soluble protein content is emphasized in some analyses made upon the bark tissue of a number of stumps of trees from which the crowns had been removed in late winter (in March). Analyses made on these tissues during June and July (as shown at the right in each of the figures) indicated, in every case, that the degree of dehardening of the cells was markedly retarded in comparison with bark tissue of normal trees, and that the content of soluble proteins, which would normally have reached a minimum in July, was still above the normal minimum and present in amounts which were equal to those present in normal trees at such times as these showed comparable hardiness. The carbohydrate content of the bark cells of the stumps were found to be as low as, or lower than, that of normal trees at any time during the year, indicating again that no direct relationship exists between degree of hardiness of the cells and their sugar contents.

On the basis of these analytical studies, made on the bark cells of some 40 locust trees collected at intervals throughout the period of one year, it is to be concluded that seasonal variations in the frost hardiness of this tissue are closely proportional to the simultaneous variations in the soluble protein content of the cells. No such correlation exists with respect to other nitrogen fractions nor with respect to soluble carbohydrate content of the cells.

It is still too early in this investigation to state definitely that the observed correlation between soluble protein content of the bark cells and their ability to resist frost injury is a causal relationship. That such a relationship actually exists, however, is strongly supported by the physiological studies mentioned above. It would appear reasonable to assume that the permeability of a cell's membrane to water or the extent to which the cell could be dehydrated or distorted without disruption of its vital organization might be dependent upon its hydrophilic protein content.

Although the correlation here demonstrated is one between protein concentration in the cells and resistance to the injurious effects of dehydration as would be produced by plasmolysis in the salt solutions used, or to the normal type of extracellular freezing, as measured by the hardiness test used, it is also possible that the increase in protein serves another purpose namely, protection of the cell against intracellular freezing in the event of rapid drop in temperature. It is conceivable in the latter case then that the higher content of soluble protein in winter protects the cells merely by inhibiting ice formation within the protoplasm by promoting undercooling so that, as cooling below the freezing point occurs, ice crystals are formed first in the intracellular spaces, the undercooled water within the cells diffuses to these extracellular crystals and no ice crystals are allowed to form within the cells. It is not yet possible to picture specifically the mechanisms involved, nor is it possible to state positively that the observed correlation is causal.

Summary

The changes which occur seasonally in the water-insoluble protein nitrogen, the water-soluble protein nitrogen, the water-soluble non-protein nitrogen, and the reducing and non-reducing sugars of the living bark tissue of the black locust are studied in relation to the seasonal variations in its frost hardiness.

Of the nitrogen fractions studied only the water-soluble proteins are found to increase in concentration in the bark in the fall along with the development of frost hardiness and to decrease in concentration in spring with the disappearance of hardiness.

Reducing and non-reducing sugars decrease in spring but more rapidly than the rate at which hardiness is lost from the tissues.

When the crown is removed from a winter tree, dehardening in the

bark of the stump is retarded and the seasonal decrease in protein, which is observed in normal trees, is also delayed in proportion to the retention of hardiness in the stump. At the same time, the reducing and non-reducing sugars in the stump decrease in concentration as in normal trees and to a greater extent.

The correlation observed between the changes in water-soluble protein and hardiness suggest that this constituent of the bark bears some causal relationship to the mechanism of development of frost hardiness. It is also indicated that the sugars are not primary factors concerned in the mechanism.

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The Chemistry of the Living Bark of the Black Locust Tree in Relation to Frost Hardiness. II. Seasonal Variations in the Electrophoresis Patterns of the Water-Soluble Proteins of the Bark [1,2]

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Introduction

In Part I of this series (1), it was shown that the concentration of the total water-soluble proteins in the living bark of the black locust tree at any time during the year was in direct correlation with the degree of frost hardiness of the cells of the bark at that time. No attempt to characterize or fractionate the proteins so obtained was described in that paper. It was considered desirable to learn, however, whether the water-soluble cell protein involved was a single protein entity or a mixture. Also, if it were a mixture, it was of interest to learn whether or not the quantitative variation with hardiness could be identified with any single component or group of components in the mixture.

The Tiselius electrophoretic technique has proved to be a useful tool in the characterization and analysis of animal proteins such as those obtained from the blood of animals (2). While some electrophoretic studies have been reported on the proteins of seeds, the cytoplasmic proteins of the vegetative parts of plants present rather an open field for investigation (3). Wildman and Bonner (4) have made a

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[2] Contents of this paper are condensed in part from a thesis presented by D. Siminovitch to the faculty of the Graduate School of the University of Minnesota in partial fulfilment of the requirements for the degree of Doctor of Philosophy, July, 1948, and in part from a continuation of the studies made under a grant from the Graduate School of the University of Minnesota.

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study of the cytoplasmic proteins of spinach leaves with the aid of electrophoresis. Aside from the interest which an electrophoretic study of the cytoplasmic proteins of bark cells would have in itself, this technique appeared particularly well suited for the kind of seasonal analysis of the proteins required in this frost hardiness study. This paper will describe the results obtained from an investigation of the seasonal changes in the electrophoretic patterns of the water-soluble cellular proteins of the black locust bark in relation to changes in frost hardiness.

Methods

The proteins were extracted from fresh shredded bark tissue with distilled water and precipitated by saturating the solution with $(NH_4)_2SO_4$. The precipitate was filtered off and dialyzed until free of sulfate against phosphate buffer of ionic strength 0.20 (consisting of a mixture of 0.19 ionic strength NaCl and 0.01 ionic strength phosphate buffer) of pH 7.2 and having a specific conductivity of 0.0115. After determination of the protein concentration in the dialyzed extracts by micro Kjeldahl analysis, they were all adjusted with buffer to approximately 1% protein concentration and equilibrated against fresh buffer overnight previous to electrophoretic analysis. The proteins were examined in the conventional Tiselius electrophoresis apparatus, utilizing the Longsworth scanning device. Scanned photographs were taken after 2 hr. runs using 50 milliamperes current with a voltage drop of 6.0 volts/cm. through the cell.

The same protein extracts were used for electrophoretic analysis as were used for determination of total extractable protein reported in the preceding paper (1) so that the values for total water-soluble protein nitrogen and for frost hardiness given in that paper apply in the present experiments.

Results

Variations in Electrophoresis Patterns between Individuals of the Species

When the location of the locust trees used in seasonal studies of frost hardiness was first selected, it was assumed that individual trees at the same stage of hardiness would not differ from each other greatly in the protein composition of their bark cells and only one or two trees were felled at each time. The results of the quantitative analyses described in the preceding paper supported this assumption.

Electrophoretic analysis soon demonstrated, however, that, to make seasonal comparisons of the protein patterns, it was necessary to restrict the comparisons to trees which were growing in closely associated groups or clones. Electrophoretic analysis showed the following rela-

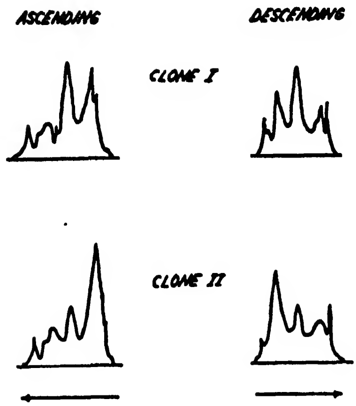

FIG. 1. The differences in Tiselius electrophoresis pattern, in winter, between the bark proteins of trees of different groups or clones of the black locust.

tionships to hold: (1) that the water-soluble protein from bark cells is a mixture of several electrophoretically distinguishable components and is not just a single protein entity; (2) that the ascending and descending patterns, while approximately symmetrical are not completely so, indicating some interaction between components in the mixture; (3) that proteins extracted from the bark tissues of different parts of the same tree give identical patterns, *i.e.*, identical ratios of the various constituent proteins in the mixture; (4) that proteins extracted from different trees cut on the same day from a group in a closely adjacent locality give closely similar patterns; but (5) the patterns obtained from the proteins of trees cut on the same day (and yielding roughly the same total amount of protein extractable) but from groups in localities as little as 100 yards apart can vary to a considerable degree from each other with respect to the relative amounts of electrophoretically distinguishable protein components present in the mixture. Each group of trees employed in this study seemed to be a clone because of underground root connections between adjacent trees. It is known that the locust can propagate vegetatively in this manner, and presumably each group or clone arose from a single seed.

The difference in the electrophoresis patterns for the proteins from individual trees collected in the winter from two clones is illustrated in Fig. 1. The characteristic pattern of the bark proteins of trees from each clone was almost exactly reproducible throughout the winter, which was the period of least seasonal fluctuation in the quantity of protein present in the bark cells.

In spite of these differences in the electrophoresis patterns as observed between proteins obtained from individuals of separate groups or clones, it was found that the seasonal variations in the total quantity of soluble proteins, as described in the preceding paper, were the same in all individuals of the black locust independent of their location. For the study of the complete cycle of seasonal changes in electrophoresis patterns occurring through the year, however, trees from a single clone had to be selected. The proteins from different trees of such a clone, consisting of trees 9–13 years of age, were determined to be so nearly identical, both with respect to total protein concentration and pattern on any one day, that any variations observed in the Tiselius patterns can be considered to constitute definite seasonal variations in the ratios of component soluble proteins of the bark cells of trees belonging to that clone.

Seasonal Variations in Electrophoresis Patterns

The Tiselius electrophoresis patterns obtained with the water-soluble proteins from bark samples collected periodically during the hardening period from July to December, 1946, are shown in Fig. 2. It is remarkable that there is little or no change in the electrophoresis patterns and,

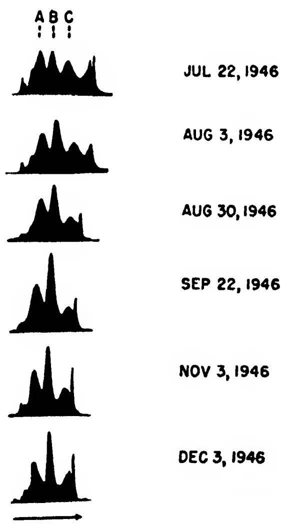

FIG. 2. Tiselius electrophoresis patterns of the soluble proteins from the bark of normal trees during the autumn hardening changes of 1946. Descending patterns only.

therefore, in the relative proportions and number of soluble protein components in the tissue during the period after early September in which the greatest change in degree of hardiness is found to occur, and in which the greatest change in the total amount of soluble protein in the tissues occurs, as described in the preceding paper. There is observed a marked change, however, during July and early August preceding or in the initial stages of the development of hardiness.

The series of electrophoretic analyses were continued into late winter, spring and early summer of 1947. As expected, the changes in the various patterns and protein fractions observed during dehardening were very much the same as in fall but in the reverse directions (Fig. 3).

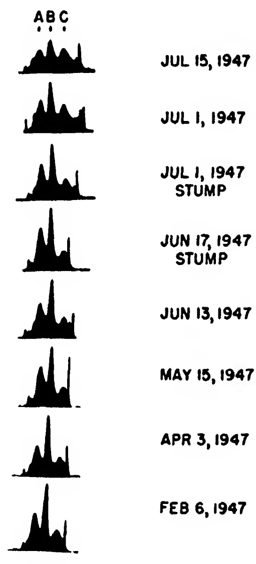

FIG. 3. Tiselius electrophoresis patterns (descending) of the soluble proteins from the bark of normal trees and stumps during the spring dehardening period of 1947.

The persistence of the same type of pattern throughout the whole winter and even in spring, after considerable dehardening and protein mobilization from the cells has occurred, is clearly in evidence. Again the most marked changes in seasonal patterns are produced during the midsummer after dehardening. The relative ratios of the various fractions of protein existing in July, 1946, are resumed to a fair degree of approximation in July, 1947.

The total area in each Tiselius pattern is proportional to the total protein concentration in the sample being electrophoretically analyzed. The proportions of the various protein fractions present can be estimated from the percentage of the total area which appears under each peak. When the percentage of each fraction is multiplied by the total amount of protein obtained from 100 g. dry weight of tissue, the actual amount of each of the various fractions in percentage of the dry weight of the tissue can be calculated.

For the purposes of reporting the relative amounts of the fractions which are present in the cell proteins, the three peaks which appear as consistently symmetrical in the patterns are designated as A, B, and C (see Fig. 2 or Fig. 3) in the order of their increasing mobilities. Material which migrates slower than A and is of variable symmetry from pattern to pattern is designated as A′ while that fraction (again of variable symmetry) which moves faster than C is designated C′. Thus, the areas of the patterns are divided into 5 fractions, 3 of which correspond to peaks of a generally high degree of symmetry. Patterns for the descending boundary were analyzed in each case. As mentioned before, the area relationship of the fractions in the ascending and the descending patterns were not completely identical. Also the limits chosen as bounding the area under each peak were those drawn to the base line from the low point between peaks. This is an arbitrary and inaccurate procedure but no other, more accurate, procedure was possible in this case. For these reasons, it is not considered that too much emphasis can be placed upon the ratios obtained except to that extent wherein broad trends are indicated.

In Fig. 4 are plotted the amounts of total water-soluble protein as found in the bark tissue at various times throughout the year, together with the amounts of each of the fractions obtained from the Tiselius patterns as described above. The actual percentages of the various fractions in the total protein are found to vary in a broadly consistent manner between the summer and winter extremes. Average percentages of the fractions A′, A, B, C, and C′ in the July total soluble protein are found to be 7, 24, 25, 26, and 17, respectively, and for the period from early September to late May, to be 6, 29, 37, 20, and 8, respectively. It is evident that, with the development of frost hardiness, the total amount of each fraction increases (see Fig. 4) but that fractions A and B increase in greater proportions than do the others. This is especially true of fraction B. In this connection it is of significance to

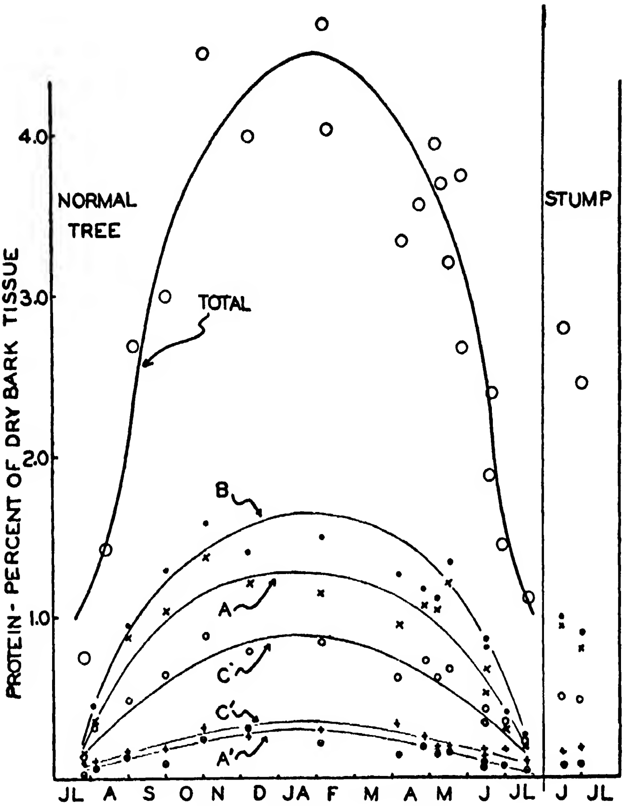

Fig. 4. Variations in the total water-soluble proteins of bark cells and in the various fractions of the soluble proteins as they occur throughout the year in normal trees and in stumps, as estimated from electrophoresis patterns.

point out that the total protein content, the distribution among the various fractions (Fig. 4) and the general contour of the patterns (Fig. 3) for the protein extracted from stump tissue (See Part I for description of this tissue) collected in July are more nearly like those of early spring normal tissue than they are like normal July tissue. This is in agreement with the finding of a retardation in loss of hardiness of this stump tissue and with the idea that hardiness is causally related to the total protein content of the tissue.

Discussion

Results of the analytical estimations of amounts of water-soluble proteins present in the bark cells of the black locust tree at various

times of year, together with the results of electrophoretic analyses of these water-extractable proteins obtained from individual trees belonging to one closely associated group, or clone, throughout the same time period, yield evidence for the following sequence of events as occurring in the bark cells curing one year's cycle in relation to the physiological states of the cells at the various periods of the year. While the actual studies reported here extended from July, 1946, to August, 1947, it is more convenient to discuss the results as covering the time period from the deep winter of one year through the spring, summer, and fall into the winter of a following year.

If we start our cycle in January, it is found that, at this period, the bark cells exhibit a maximum of frost hardiness, contain a maximum percentage of their dry weight in the form of water-extractable protein which, by electrophoretic analysis, yields a characteristic pattern in which the percentage amounts of the 5 fractions A′, A, B, C, and C′ are approximately 6, 29, 37, 20, and 8, respectively. These conditions are maintained without detectable change through February and March, although dormancy is broken sometime after the month of January. In April, sap begins to rise in the tree. By early May the buds have begun to swell, followed by leaf emergence during May, and growth of leaves to full size by the last of June. During the period from late April to the middle of June there occurs, in the bark cells, a rapid drop in water-soluble protein content with an accompanying and proportional drop in frost hardiness of these cells. During the early part of this drop in total water-soluble protein content, it is found that very little change in the percentage composition of this protein mixture occurs (in terms of the arbitrarily chosen fractions), all components being mobilized from the cells in about the same proportions. During the latter part of May and the early part of June, however, in the period of most active leaf growth, the total protein content drops rapidly, and it becomes apparent that a shift in percentage of the various components of that protein which remains in the bark cells is taking place. Also, at this period is observed the beginning of new growth of the bark. Cambial growth becomes increasingly rapid. By early July, leaf growth has ceased, cambial growth has reached a maximum rate and begins to fall off in rate. At this time, the content of water-soluble proteins in the bark cells is at a minimum, frost hardiness is at a minimum, and the percentage amounts of the 5 fractions detectable by electrophoretic analysis have changed to the extreme

difference from those of winter tissue. At this time the percentages of A′, A, B, C, and C′ fractions are approximately 7, 24, 25, 26, and 17, respectively.

In late July, cambial division is terminated and the new annual ring of wood has been virtually completed for the year. Final lignification of the wood then proceeds during August and the bark cells begin to prepare themselves for winter. By early August their protein content is already increasing rapidly, the maximum being approached at about the time the leaves are lost in the middle of October and attained in November. Maximum frost hardiness is reached at this time. The electrophoresis pattern has become nearly characteristic of winter proteins by the latter part of August, and this pattern is then maintained throughout the winter and the cycle is complete.

These electrophoresis observations were made on the proteins of the bark of trees belonging to what appears to be a single clone. A study of the complete cycle of patterns seasonally within other clones has not yet been made. Some electrophoretic analyses of the proteins from winter bark of trees belonging to a second clone have led to the unexpected conclusion that a variation exists in the ratios of the component proteins of bark cells within the species, black locust. The patterns of the two clones were compared in winter because that seems to be the period of least seasonal change. It is probable that the differences in the respective patterns of the two clones can be accounted for by differences in the relative proportions of the various protein fractions that make up the total soluble cytoplasmic proteins rather than by differences in the nature of the protein fractions, although no attempt has been made to separate the fractions by solubility procedures. These differences in protein patterns in winter between groups, or clones, of trees are consistent and are not reflected in any apparent pathological symptoms in one or other of the groups or in any morphological difference. This is interesting because very little variation has been observed in the Tiselius patterns of normal human blood proteins, and even small deviations from the normal reflect pathological conditions (5).

No taxonomical study has been attempted to determine whether different varieties of the species were involved in the study. Also, it has not been established that the differences observed between groups of trees are not environmental. However, if environment were responsible, it is difficult to see how the characteristic pattern is so closely

maintained from tree to tree within a group or clone and reproduced from year to year, without some small variation in response to some local environmental condition. Underground root connections were clearly in evidence between some of the trees in each group which led to the belief that each group is a clone. Apparently, the characteristic protein patterns of each clone are due to genetic differences and are transmitted vegetatively. This would warrant further investigation in which the Tiselius patterns of the proteins of individual plants of the same age and grown in the same environment, but derived from different seeds, would be compared.

Summary

A seasonal study of the water-soluble cytoplasmic proteins of the bark of the black locust tree by electrophoretic analysis is made in relation to its frost hardiness.

The extractable proteins consist of at least 5 major electrophoretically distinguishable components.

During the periods of the greatest changes in hardiness and in concentration of the total protein, in fall and spring, no significant deviation occurs in the electrophoresis patterns from that which is characteristic of the winter protein. Marked changes occur in the pattern in midsummer, however. These changes are produced by relatively large shifts in the concentrations of 2 or 3 of the components relative to the other components of the total protein and not in the number of electrophoretically detectable components present.

When the crown of a tree is removed, the normal development of the electrophoresis pattern characteristic of summer tissue proteins are somewhat retarded in the proteins of the stump. This retardation coincides with the inhibition of dehardening and of protein hydrolysis in the stump tissue.

On the same day the electrophoresis patterns of the proteins taken from trees growing in close proximity, or within a "clone," are essentially identical, as are the patterns of proteins taken at various levels on the same tree.

The electrophoresis patterns of proteins taken on the same day from different trees in more widely separated groups or "clones" are not identical. These differences seem to be accounted for by differences in relative concentrations of the various components of the total protein.

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On the Nature of Highly Purified Mushroom Tyrosinase Preparations

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Introduction

In previous communications from this laboratory (1,2,3), two types of highly purified mushroom tyrosinase have been described. Whereas both types of tyrosinase possess the ability to catalyze the aerobic oxidation of both monohydric and *o*-dihydric phenols, the ratio of these two activities is markedly different in the two types of preparations. Tyrosinase preparations whose ratio of activities (catecholase to cresolase)[2] is relatively high have been called high catecholase preparations, and those in which the ratio of the catecholase to cresolase activity is relatively low have been called high cresolase preparations.

Much effort has been directed in the past toward ascertaining whether or not the two activities of tyrosinase are those of one enzyme or a mixture of two enzymes, but the results thus far have not led to a satisfactory conclusion. The problem is a fundamental one because of the probable role of tyrosinase in plant respiration and other natural processes.

Attempts have been made previously in this laboratory to obtain further information concerning the physical nature of tyrosinase by means of electrophoresis. Preliminary experiments were carried out using crude mushroom tyrosinase preparations, for it was known that the ratio of activities could most easily be changed by chemical means when crude preparations were employed. The results of these preliminary experiments led to the conclusion that no clean cut separation of

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[2] The activities of the enzyme tyrosinase in catalyzing the aerobic oxidation of the monohydric phenol, *p*-cresol, and the *o*-dihydric phenol, catechol, are referred to as cresolase and catecholase activities, respectively.

the two activities could be brought about in crude preparations by means of electrophoresis. It is the purpose of this communication to report on the results obtained using highly purified enzyme preparations,[3] and from the results to propose a model that appears to reconcile the conflicting views concerning the nature of the enzyme tyrosinase.

Enzyme Preparations

Five highly purified tyrosinase preparations made from the common mushroom *Psalliota campestris*, were used in this investigation. They were prepared by methods developed over a number of years in this laboratory, involving repeated fractional salt precipitations and alumina adsorptions. Details of the preparative procedures are published elsewhere (4,5). The copper content and activity data on the 5 preparations used are summarized in Table I. It can be seen from the activity ratio data in the table that 4 of the preparations were of the high catecholase type. It was originally planned to examine electrophoretically

TABLE I

The Enzymatic Properties and Copper Contents of 5 Highly Purified Tyrosinase Preparations from the Common Mushroom, Psalliota compestris

Preparation[a]	Copper[b]	Activity[b] units/ml.		Units/γ Cu		Units/mg. dry weight		Activity ratio
		Cat.	Cre.	Cat.	Cre.	Cat.	Cre.	Cat./Cre.
	per cent							
I	0.206	21,500	450	2130	48	4400	95	48
II	0.204	49,000	1140	1980	47	4050	95	43
III	0.103	11,500	570	2100	104	2150	107	20
IV	0.098	18,500	1100	2320	137	2270	135	17
V	0.028	1,880	1180	856	536	237	149	1.6

[a] For simplicity of discussion in this paper, the enzyme preparations have been assigned numbers different from those used in the original data and published elsewhere (5). The number key is as follows:

I = C211-228F2II, II = C211-228F2I, III = C175BI, IV = C172-5AB, V = C189.

[b] For methods used in measuring the activities and copper content see experimnetal section.

[3] Certain of the results of the electrophoretic investigation of crude and purified mushroom tyrosinase preparations have been briefly described earlier in a review article on tyrosinase (3) and referred to as *unpublished work.*

more than one purified preparation of each type. However, due to preparational difficulties it was possible to include only one purified high cresolase preparation.

Before considering the results of the electrophoresis studies, it will be helpful to study in some detail the data given in Table I. It will be observed that the preparations listed in Table I are arranged in the order of decreasing copper content. Also, it is worthy of note that this order of arrangement corresponds to an increase in the units of cresolase activity/γ of Cu as seen in Col. 6 of the table. The constancy of the catecholase activity/γ of Cu for the high catecholase preparations I to IV (Col. 5) confirms the dependence of the catecholase activity on the copper content as previously reported by Keilin and Mann (6) and Ludwig and Nelson (1).

Using units of activity/mg. dry weight of protein as a measure of the purity of the enzyme, it can be seen from Col. 7 of Table I that the "catecholase purity" decreases with a decrease in copper content. On the other hand the "cresolase purity" increases with decrease in copper content (see Col. 8). This relationship between purity and copper con-

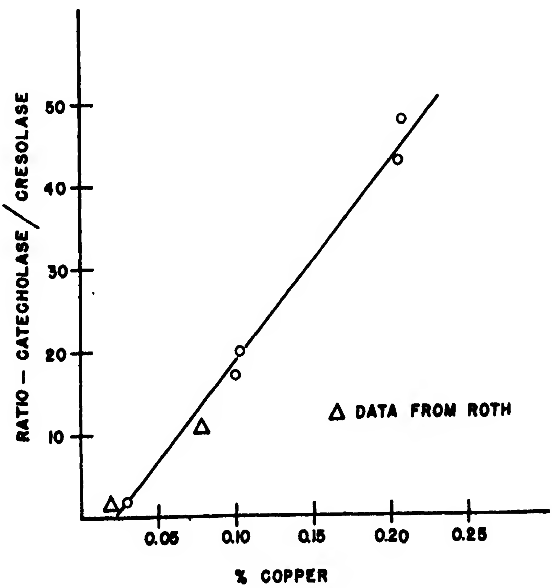

Fig. 1. The relationship between the activity ratios and copper contents of highly purified tyrosinase preparations. The copper data on the two preparations taken from Roth (7) were obtained by the manometric method of Warburg and Krebs (8). For other details see section on methods.

tent suggests the presence of a copper-free protein possessing cresolase activity as a contaminant in preparations III, IV, and particularly V.[4] However, such an explanation of the data is not the only one possible; indeed, as will be pointed out subsequently, there are reasons for favoring another interpretation.

Before leaving Table I, one additional comparison of data should be made, namely, that the activity ratio and the copper content change in the same direction. Furthermore, when plotted (see Fig. 1), a linear relationship between these two quantities is suggested. The significance of this observation will be considered later.

Electrophoresis

The 5 purified tyrosinase preparations described in Table I were electrophoretically analyzed in a small volume electrophoresis apparatus of the Tiselius type equipped to analyze the migrating boundaries optically[5] (for experimental details, see legends of Figs. and Tables). The studies were made with 3 purposes in mind. In the first place, a criterion of the homogeniety of these preparations was desired. Secondly,

TABLE II

Showing How the Electrophoretic Mobility of the Main Component in Tyrosinase Preparations of High Electrophoretic Homogeneity is Related to the Activity Ratio of Such Preparations[a]

Preparation	Ratio $\frac{\text{Cat.}}{\text{Cre.}}$	$\mu \times 10^{5}$ (Main component)	Homogeneity	pH	Buffer (primary-secondary phosphate)
			per cent		
I	48	6.38	100	7.58	0.05 *M*
II	43	6.32	100	7.67	0.05 *M*
III	20	5.3	95	7.71	0.10 *M*
IV	17	4.5	93	7.58	0.15 *M*
V	1.6	3.0	70–80	7.71	0.10 *M*

[a] Unfortunately, only preparations III and V were run at the same ionic strength (0.28), and the mobility values for preparations I, II, and IV should probably be corrected to this same ionic strength for a better comparison. Such a correction (see Abramson, Moyer and Gorin, Electrophoresis of Proteins, Reinhold, 1942, p. 164, Eq. 8) yields mobility values of 5.21, 5.16, and 5.05 $\times$ 10^{-5} for preparations I, II, and IV, respectively. The corrected values are plotted as a broken line in Fig. 2.

[4] On the basis of copper content and "catecholase purity" it would appear that the high catecholase preparations III and IV were only about half as pure as the high catecholase preparations I and II.

[5] All electrophoresis and ultracentrifuge experiments were performed by Dr. D. H. Moore, College of physicians and Surgeons, Columbia University.

if tyrosinase should be a mixture of two enzyme proteins, a method of separation was needed whereby the monophenolase activity alone could be recovered. And finally, if tyrosinase should be a single protein enzyme, a knowledge of the surface properties (as indicated by the mobility[6]) of these purified preparations might yield some information regarding the nature of the enzyme molecule.

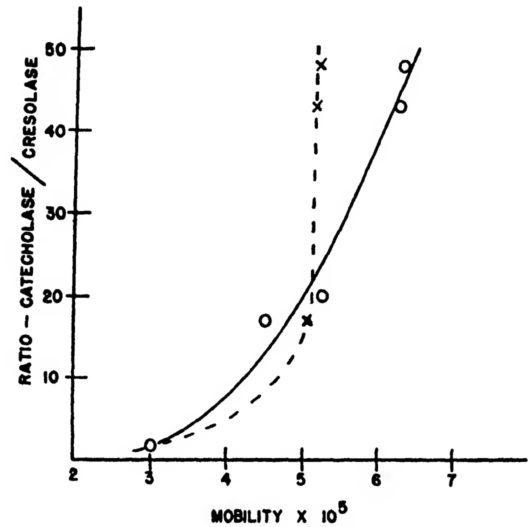

FIG. 2. The relationship between the activity ratios and electrophoretic mobilities of highly purified tyrosinase preparations. Measurements were taken with the small-scale Tiselius apparatus at pH values from 7.58 to 7.71 at 1°C. See footnote to Table II for significance of broken line curve.

Table II contains electrophoretic mobility and homogenity data at about the same pH value for the 5 purified preparations presented in Table I. The relationship between the mobility of the main component and the activity ratio is shown graphically in Fig. 2 (see footnote, Table II). The homogeneity data in Col. 4 of the table, estimated from scanning diagrams of the moving boundaries (such as are produced in Fig. 3) show that, for preparations III, IV, and V, significant amounts of an "impurity" can be electrophoretically resolved. In all 3 cases this material migrated more rapidly than the main component. Preparations I and II, however, were completely homogeneous within the limits of the electrophoretic method.

In cases of preparations III, IV, and V, all of which contained more than one component, the electrophoresis experiments were prolonged to permit the partial separation of the components. By means of a motor-

[6] The electrophoretic mobility of a protein is largely dependent on its surface properties. See H. A. Abramson, L. S. Moyer and M. H. Gorin, Electrophoresis of Proteins. Reinhold, New York.

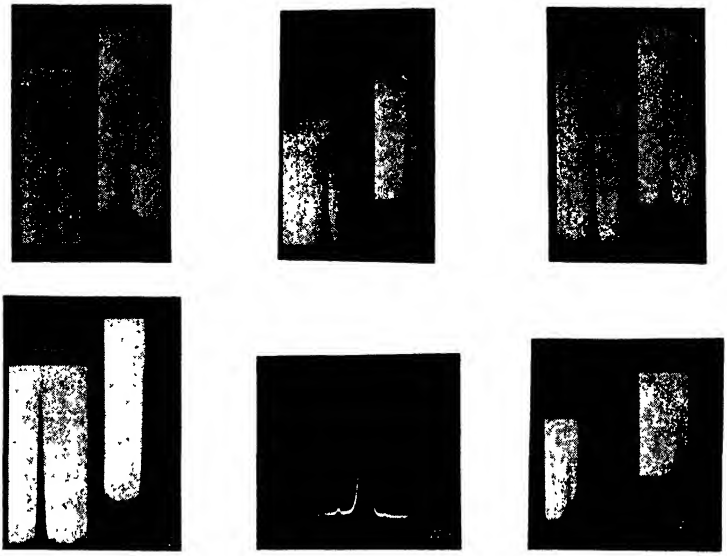

FIG. 3. Scanning diagrams made during the electrophoresis of 5 different highly purified tyrosinase preparations (I, II, III, IV, and V) in the small-scale Tiselius apparatus. All diagrams except IVa show both the ascending (left) and descending (right) boundaries taken by the Longsworth scanning method (9). Diagram IVa (ascending boundary only) was made on preparation IV using the Philpot-Svensson (10,11) scanning method. Each diagram shows the homogeneity of the protein boundaries after migration in an electric field for a given time interval. The distance of migration can be estimated for each component by comparing the position of each peak with the boundary position before the electrophoresis was started.[a] In diagrams I, II, III, and IV the initial position of the boundary is recorded as a slight break in the white line just above each scanning diagram. This white line is a photograph of the slit taken before the electrophoresis was begun.[b] All of the experiments were carried out at 1 ± 0.01°C. using primary-secondary phosphate buffers. The constant for the conductivity cells, used to measure the resistance of the buffers and buffered protein solutions, was 1.089 reciprocal cm. Other data pertinent to each experiment are listed in the table below.

Diagram	I	II	III	IV	IVa	V
Time, hr.	1	1	1.5	3	23	3
pH	7.58	7.67	7.71	7.58	7.55	7.71
Buffer, conc. *M*	0.05	0.05	0.10	0.15	0.15	0.10
Resistance of buffer and protein	291	293	163	116	116	161
Current milliamps.	18	18	26	30	50	28
Voltage between electrodes	170	180	120	120	100	120
Cross-section area of cell, cm.^{2}	0.761	0.761	0.800	0.761	—	0.761
Magnification factor	0.560	0.645	0.560	0.645	—	0.645

[a] Except for diagram IVa and V.

[b] The scale of distance of migration along the abscissa of diagram IVa is different from that of IV.

driven hypodermic syringe, samples were then taken of the main components, the fast components, and the unseparated middle portions corresponding to the original enzyme preparations. In every case the enzymatic properties of the main components were the same as the properties of the respective original preparations. As was also expected, the unseparated middle portions were the same as the originals.

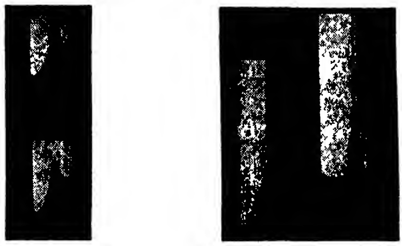

FIG. 4. View A represents sedimentation diagrams for preparation I in an air-driven ultracentrifuge taken by the Longsworth scanning method. The experiment was conducted using 0.05 *M* primary-secondary phosphate buffer pH 7.58 at 32°C. The time interval between the upper and lower diagrams was 10 min. with the cell making 790 r.p.s. about an axis 57.06 mm. from the bottom of the cell. Reference lines appear as very faint streaks at the edges of the diagrams. Two other diagrams (not shown) were made at earlier times. Ten to 20% of a lightweight material is shown at the right of the main component.

View B is a diffusion diagram taken with preparation I in a small-scale Tiselius electrophoresis apparatus using the Longsworth scanning method. The experiment was carried out in 0.05 *M* primary-secondary phosphate buffer pH 7.58 at 1 ± 0.01°C. These views of the diffusing boundaries in both legs of the U-tube were taken 23 hr. 8 min. after beginning the experiment. Other diagrams were taken up to a diffusion time of 48 hr. The magnification factor of the lens system was 0.645.

The samples of the fast components all contained only traces of enzyme as evidenced by activity measurements. Furthermore, the properties of the activities and the activity ratios were the same as for the pure main components. It seems likely, therefore, that the pure fast components actually possessed no tyrosinase activity, but that the samples all showed a very slight activity as a result of mixing during the sampling process. From these studies it may be concluded that no electrophoretic separation of enzyme activities took place in any of the 5 highly purified preparations investigated, and the electrophoretic homogeneity of the high catecholase preparations was very high under the conditions employed.

Mobility data are recorded in Table III as taken with preparations III and IV at various conditions of pH. The experiments leading to these results were performed in the hope of ascertaining the isoelectric point of tyrosinase. Although no definite conclusion can be drawn from the data of Table III, it is certain for preparations III and IV that the isoelectric point is below pH 5, probably in the vicicity of pH 3 or lower. Of importance in such studies is the fact that tyrosinase starts to lose its Cu and enzyme activity in solutions buffered below pH 5; and, therefore, electrophoresis investigations are restricted to pH values near 5 or above. There is some reason to believe that the isoelectric point may vary with the activity ratio of the enzyme.

TABLE III

Showing the Dependence on pH of the Electrophoretic Mobility of the Main Component of Two Highly Purified Mushroom Tyrosinase Preparations

Preparation	Ratio $\frac{\text{Cat.}}{\text{Cre.}}$	$\mu \times 10^5$ (Main component)	Homogeneity	pH	Buffer
			per cent		
III	20	5.3	95	7.71	0.1 *M* primary-secondary phosphate
		4.8	97	5.86	McIlvaine
IV	17	7.9	90	8.9	Sørensen borate-HCl
		4.5	93	7.58	0.15 *M* primary-secondary phosphate
		2.5	95–100	4.97	McIlvaine

Kubowitz (12) reported that purified potato tyrosinase was isoelectric at pH 5.4. Experience in this laboratory has shown that the potato enzyme resembles the mushroom high cresolase preparation more closely than it does the high catecholase enzyme. It seems possible, therefore, that a high cresolase preparation might have a higher isoelectric point than suggested for the high catecholase preparations of Table III.[7]

Since it is known that two substances may have fairly similar electrophoretic mobilities and yet differ greatly in other respects, an additional criterion of the purity of these tyrosinase preparations was desired. The ultracentrifuge provides one such criterion, since it is capable of resolving particles which differ in their sedimentation constants. Unfortunately, however, only preparation I was available in sufficient quantity for both electrophoretic and ultracentrifugal analysis.

The data obtained in the ultracentrifuge[5] using preparation I yielded a sedimentation constant for the main component of 6.4 Svedberg units at 20°C. A measurement of the diffusion constant at 1°C. gave a value of 6.1×10^{-7} when recalculated to 20°C.

[7] It is unfortunate that not enough of the high cresolase preparation V was available for a determination of its isoelectric point.

Using these values and assuming a partial specific volume of 0.75 cm.^{3}/g. [an average for many proteins (13)], the molecular weight of the tyrosinase molecule in preparation I calculated to be 102,000 with an uncertainty of about $\pm 10\%$.

An examination of the plate reproduced in Fig. 4 [a photograph of the sedimenting boundary taken by the Longsworth scanning method (14)] reveals that 80–90% of preparation I was present as a single homogeneous component. The impurity, consisting of 10–20% of the total, appears to be heterogeneous and is lighter than the main component. Ultracentrifugation was continued in this experiment until the main component was precipitated and could be collected as a solid. The cell contents were then removed and assayed for enzyme activity. The relatively unsedimented lighter portion was found to contain less than 1% of the enzyme activity with the activity ratio the same as for the original. Resuspension of the precipitated main component yielded a solution whose enzymatic properties were the same as for those of the original preparation. Hence, although the preparation was not homogeneous in the ultracentrifuge, it may be concluded that the two activities can probably not be separated by means of the ultracentrifuge.

A calculation based on the diffusion constant and the electrophoretic mobility indicated that the net charge on the enzyme molecules of preparation I (assuming spherical symmetry) was 17.8 electron units at pH 7.78. A negative sign for the net charge may be deduced from the fact that the enzyme migrated toward the anode. The assumption of spherical symmetry may not be justified for the tyrosinase molecule. As a matter of fact, the sedimentation and diffusion constants are in agreement with some deviation from the spherical form. This is revealed by a calculation of the molecular frictional factor, which in this case is 1.26, corresponding to an axial ratio of about 5.5, assuming no hydration of the molecule. On the other hand, proteins are commonly hydrated and the apparent asymmetry may be due in considerable part to such hydration. Adair and Adair (15) suggest that an asymmetry of the magnitude calculated above cannot be exclusively due to hydration and some must be inherent in the molecule.

Discussion

As indicated in the foregoing, preparation I was 80–90% homogeneous in the ultracentrifuge and had a molecular weight of about 100,000. Assuming that the heterogeneous impurity contained no copper, then the copper content of the molecule can be revised from 0.206% upward to about 0.25%, a value which corresponds to 4 Cu atoms per enzyme molecule. To justify the assumption that the impurity contained no copper, it should be recalled that the catecholase activity of this preparation bore about the same relationship to copper as was observed for the other high catecholase preparations (see Table I). Furthermore, the parallelism between the catecholase activity and copper content during many stages of purification indicates that the impurities are copper-free and that all non-enzyme copper has been removed.

It will be recalled that a consideration of the copper content data and "catecholase purity" data shown in Table I lead to the suggestion that preparations III and IV might be only about half as pure as preparations I and II by reason of contamination with copper-free protein. To explain the increase in "cresolase purity" with decrease in copper content from Preparation I to V, it was only necessary to ascribe cresolase activity to the copper-free protein. However, as shown in Table II, preparations III and IV were found to be over 90% homogeneous using electrophoresis as a criterion, rather than about 50% pure as suggested by the reasoning above. Preparation V was found to be 70–80% homogeneous rather than less than 15% homogeneous as suggested on the basis of copper content. Furthermore, no evidence for the existence of a cresolase protein was found on enzymatic examination of the relatively small amounts of other protein components separated from the main components of each of these preparations during electrophoresis.

As illustrated by the situation encountered with preparation I, where 10–20% of lightweight material was found in the ultracentrifuge, although this preparation appeared homogeneous by electrophoresis, it might be argued that a relatively large amount of copper-free non-enzyme material might be present in preparations III, IV, and V and not be electrophoretically distinguishable. Such a possibility seems rather remote, however, since the data in Table III reveal that a considerable shift in the pH of electrophoresis did not greatly alter the observed homogeneities of preparations III and IV. It would be very unusual for two proteins to have the same mobility-pH characteristics over a pH range from 5 to 9. Moreover, the assumption involving an electrophoretically indistinguishable protein component does not satisfactorily explain the relationship observed (see Fig. 2) between the electrophoretic mobility of the main component and the activity ratio; a fact which can be due only to a change in the surface characteristics of the tyrosinase molecule. It would seem, therefore, that the physical, analytical and activity data on these 5 purified preparations cannot be accounted for on the basis of the presence of more than one protein component.

It is possible, however, to account for these and other experimental tyrosinase data on another basis; a basis involving quite a new concept of the tyrosinase molecule. Before presenting this concept or model, however, it will be helpful to list certain important facts that have now

been established in regard to the nature and mode of action of highly purified tyrosinase preparations. These facts may be summarized as follows:

1. Crude mushroom tyrosinase preparations possess the ability to catalyze the aerobic oxidation of both monohydric and *o*-dihydric phenols. The two activities, which appear to involve quite different reactions, are referred to as cresolase and catecholase activities, respectively (3).

2. The ratio of catecholase to cresolase activity may be readily increased during purification (1,6,12,16) and the activity ratio of partially purified preparations may be changed by mild physical treatments such as warming (16,17), treatment with charcoal (18), and by adsorption on kaolin or alumina (16).

3. In purified preparations possessing a high catecholase to cresolase activity ratio (high catecholase preparations) only the catecholase activity is proportional to the copper content (1,6,12), and such preparations have properties differing from those of the enzyme as it occurs in the mushroom juice. In purified high cresolase preparations both activities are proportional to the copper content (2,19) and the properties of such preparations more closely resemble the properties of the enzyme in the plant juice.

4. A change in the activity ratio is always accompanied by a change in the purity level of both activities (cresolase and catecholase activities/mg. dry weight) and in highly purified preparations the activity ratio and the copper content are related (see Fig. 1).

5. A tyrosinase preparation having only monophenolase activity has never been reported (3) and the simultaneous oxidation of *o*-dihydric phenol is necessary for the enzymatic oxidation of monohydric phenol (20,21).

6. Both activities are inhibited to the same extent by the copper inhibitors, cyanide and diethyldithiocarbamate (22).

7. The electrophoretic mobility varies with the activity ratio (see Table II and Fig. 2.) and neither electrophoresis nor ultracentrifugation separate the two activities.

8. Several purified preparations differing widely in copper content and activity ratio have been found to possess a high degree of homogeneity (see Tables I and III).

A New Model for the Enzyme

The several hypotheses involved in the construction of an enzyme model that reconciles all of the above tyrosinase data, stem from the observation made in this study that several highly purified tyrosinase preparations having different copper contents and different activity ratios were each judged to possess a high degree of homogeneity and activities that could not be separated. These conditions lead to the fundamental hypothesis of the model to be proposed, *i.e.*, the molecular sizes of the enzyme molecule are different in tyrosinase preparations having different activity ratios, but the number of copper atoms per enzyme molecule is essentially constant.

To account for the relationships between the copper contents, molecular weights, and activity ratios of various types of tyrosinase preparations, another important hypothesis of the model states that a fragmentation or loss of portions of the natural tyrosinase molecule occurs as the result of chemical or physical treatment during the preparation or purification of the enzyme solution. As a third and final postulate, the model requires that the fragments lost during the preparative procedures contain none of the copper originally present in the natural molecule, but such fragments must be in some way important elements in the cresolase activity. It also seems likely that, during the early stages of the fragmentation process, the catecholase activity is influenced. Furthermore, a loss of fragments of the molecule would be expected to alter the net charge and therefore the electrophoretic mobility of the enzyme due to removal, alteration or formation of new charged groups. As previously shown (Fig. 2) a relationship between the mobility and the activity ratio was observed.

Suggesting that the size of the tyrosinase molecule varies throughout the preparations listed in Table I amounts to proposing a higher molecular weight for high cresolase than for high catecholase preparations. Unfortunately, it was only possible in this study to determine the molecular weight of the high catecholase preparation I (molecular weight 100,000). However, Tenenbaum (17) determined the molecular weight of the enzyme in a mushroom tyrosinase preparation by the diffusion method of Northrop and Anson (23) and reported a value of about 200,000. The preparation used by Tenenbaum apparently had a considerably lower activity ratio than preparation I, since the described enzymatic properties resemble those of preparations III and IV. No

data on the copper contents were included in Tenenbaum's report. However, on the basis of the model presented above, a preparation having a lower activity ratio than preparation I would be expected to have a higher molecular weight.[8]

If it be assumed, as in the model, that the natural tyrosinase molecule is a single protein complex possessing 2 characteristic activities and containing 4 atoms of copper, and the size of the molecule decreases during the process of obtaining a high catecholase type preparation, and the cresolase activity is associated with the fragments lost in this process (as well as on the copper), then the facts previously enumerated as applying to the nature of tyrosinase, may be explained as follows: (The points are numbered to correspond with the previous summarization.)

1. The fundamental definition of the enzyme model as stated above is based on a single protein entity possessing two different activities.
2. A change in the activity ratio on treatment of the molecule in various ways would arise from the partial loss of portions of the molecule responsible for the cresolase activity as well as from the "unmasking" of the "catecholase centers" as the large high cresolase type molecules decrease in size.
3. In a series of high catecholase preparations the catecholase activity would be proportional to copper while the cresolase activity would not be proportional owing to the loss, in varying amounts, of the groups involved in the catalysis of *p*-cresol oxidation. In high cresolase preparations both activities should be more or less proportional to copper with a lowered value for the catecholase activity/γ of Cu. A decrease in the catecholase activity per unit of Cu might be expected for large molecules where some "catecholase active centers are masked" by the large size of the molecule.
4. The catecholase activity/mg. of dry weight (catecholase purity)

[8] The authors are fully aware of the fact that the proposed model for the enzyme tyrosinase needs further experimental evidence that the molecular size of the enzyme actually does vary with the activity ratio. Such experiments will be performed as soon as possible. Due to the great difficulty of preparing sufficient amounts of reasonably homogeneous tyrosinase specimens of relatively low catecholase/cresolase ratio it is likely that some time may elapse before the obvious ultracentrifuge and diffusion experiments can be performed. In the meanwhile, it is hoped that the proposed model will stimulate research on this important enzyme and lead to a better understanding of its action.

would increase with increasing activity ratio due to a decrease in molecular weight without loss of catecholase activity. The "cresolase purity" might increase, decrease, or remain constant, depending on the relative rates of change in molecular size and cresolase activity per molecule. According to Table I there is actually a slight decrease with increasing ratio. As protions of the molecule or complex are lost, the percentage copper content would increase, always providing that the fragments lost contain relatively little copper.

5. By definition, the model precludes the possibility of a separate cresolase enzyme. The cresolase activity is associated with parts of the molecule which when separated as fragments possess no enzymatic (cresolase) activity. The close association of the two activities in the model is in line with the observation that the simultaneous oxidation of *o*-dihydric phenol is necessary for the enzymatic oxidation of monohydric phenol.

6. Since, in the model, both activities are dependent on the same Cu atoms, substances which combine with Cu would necessarily inhibit both activities to the same extent.

7. Electrophoretic properties of the molecule might be expected to change as fragments are removed from the molecule, since it is probable that both the diffusion constant and the net charge would change.

8. According to the model, highly purified tyrosinase preparations (of either type) should possess the properties of a single homogeneous protein entity.

Experimental

The experimental details associated with the electrophoresis studies are given in the legends of the several tables and figures.

Three different methods were employed to determine the copper content of the several enzyme preparations. Preparations I, II, and V were assayed by the polarographic method of Ames and Dawson (24). Preparations III, IV, and V were assayed by the manometric method of Warburg and Krebs (8), based on the copper catalysis of the oxidation of cysteine. Preparation IV was also analyzed spectrographically.[9]

It will be noted that the copper of preparation V was determined in two ways. The cysteine method yielded 0.0296% and the polarographic method 0.0255%. The preparation of the copper-free water and other precautions found necessary have been previously described (24).

[9] Determination made by Dr. A. F. Daggett of the University of New Hampshire who reported 0.095% copper compared to 0.098% found by the Warburg-Krebs method. No metals other than copper were found except for an estimated 0.5% of calcium.

Catecholase activity was determined by the chronometric method (25) and cresolase activity was assayed manometrically (26). Protein concentrations were estimated as dry weights of undialyzable solids (5).

Acknowledgments

The authors gratefully acknowledge the helpfulness of preliminary experiments on the electrophoresis of crude tyrosinase preparations carried out in this laboratory with Dr. Stanley R. Ames, now of Distillation Products, Inc., Rochester, New York. The authors are also indebted to Mr. Stanley Lewis of these laboratorise for aid in the preparation of the enzyme specimens, to Dr. Albert F. Daggett of the University of New Hampshire for the spectrographic analysis, and to Dr. Daniel H. Moore of the College of Physicians and Surgeons, Columbia University for the electrophoretic and ultracentrifuge analyses. The preliminary phases of this investigation were made possible by a grant from the American Philosophical Society.

Summary

1. Five highly purified tyrosinase preparations obtained from the common mushroom, *Psalliota campestris,* and possessing different amounts of copper, different ratios of catecholase to cresolase activities, and different levels of purity as indicated by the activities per mg. of dry weight, have been electrophoretically analyzed in an apparatus of the Tiselius type. One preparation was also examined in the ultracentrifuge.

2. No separation of the two activities of tyrosinase was effected by the electrophoresis and ultracentrifuge procedures. Purified preparations of markedly different copper content and activity ratio were found to possess equally high orders of electrophoretic homogeneity.

3. On correlating the activity, copper and homogeneity data, it has been found possible to explain all of the known properties of mushroom tyrosinase in terms of a single copper-protein entity. The principal feature of this explanation is that different types of tyrosinase (different activity ratios, *etc.*) arise as the result of fragmentation of the protein molecule during the preparative procedures.

4. A model of the tyrosinase molecule is suggested, which accounts for a decrease in the ability of the enzyme to catalyze the oxidation of *p*-cresol and an increase in the copper content as the size of the molecule is decreased during treatment in various ways. The model also accounts for the proportionality between the catecholase activity and copper content after preliminary stages of purification. Although additional experimentation is necessary to prove definitely the validity of the model, all tyrosinase data now available are in agreement.

5. The ultracentrifuge results indicate that purified mushroom tyrosinase of the high catecholase type is a copper protein of molecular weight about 100,000 and containing 4 atoms of copper/molecule (0.25% Cu).

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Separation of a Crystalline Globulin from Tomato Juice and Determination of Its Isoelectric Point [1]

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Introduction

In connection with the separation of a new food accessory related to thiamine metabolism (4), we have had occasion to isolate a crystalline globulin from tomato juice serum (*Lycopersicon esculentum*) and determine its isoelectric point. It was necessary to know whether the globulin behaved as a cation or anion, so one could know whether it would remain in solution or come out on the cation exchange resin employed to separate the new food accessory.

Many of the plant globulins (1) have an isoelectric point around pH 5.2–5.5. However, we found that asclepain from milkweed had an isoelectric point of pH 3.11 (2). The globulin from tomato has an isoelectric point of pH 3.43 as reported herein.

Experimental

Preparation of Tomato Globulin

Fresh juice from John Baer tomatoes was run slowly through a Sharples supercentrifuge to remove the red cellular debris and finally filtered crystal clear through asbestos and paper pulp. The filtered juice was light yellow in color and had a pH of 4.2 and contained 4.5% total solids, the bulk of the latter being sugars. The juice was concentrated to 35–40% solids by freezing out ice in 5–6 successive freezings, separating ice from the mother liquor each time in a perforated bowl centrifuge and refreezing the mother liquor. The concentrated syrup was subjected to dialysis against water. The dialyzate contained the new food factor as well as sugar and other dialyzable substances. The globulin precipitated out inside the dialyzer in amorphous condition and brownish in color. This was transferred to cylinders and sedimented in a

[1] Approved by the Director of the New York State Experiment Station for publication as Journal Paper No. 768.

centrifuge. The globulin was dissolved in warm (50°C.) M NaCl solution, stirring occasionally for 2 hr., and the liquid separated from undissolved matter in a centrifuge. The somewhat turbid centrifugate was filtered crystal clear through paper pulp and asbestos, diluted with 5 volumes of water at 50°C. and again filtered through paper pulp and asbestos. Toluene was added and the globulin solution was placed in the cold room at 0°C. for 48 hr. to crystallize. The mother liquor was decanted off and the colorless crystalline globulin separated in the centrifuge. The globulin was redissolved in warm M salt solution, placed inside a dialyzer tube and attached to a slowly driven stirrer which rotated dialyzer tube and contents in a beaker of conc. $(NH_4)_2SO_4$ solution. As the concentration of $(NH_4)_2SO_4$ increased within the dialyzer tube, solid $(NH_4)_2SO_4$ was added to the outer solution. Small, needle-like crystals of the globulin began to separate, and after 3–4 days the operation was concluded and the crystals separated centrifugally from the mother liquor. The crystals were redissolved in warm M salt solution, filtered and recrystallization carried out by 5-fold dilution with water as above. The crystals were finally suspended in distilled water and electrodialyzed between parchment membranes to remove the last trace of salt. This suspension was preserved with toluene in the cold room until used in the electrophoresis work. From 40 gal. of tomato juice some 5–6 g. of globulin were separated.

Electrophoresis Experiments

Preliminary electrophoresis experiments indicated that the isoelectric point of the globulin lay between pH 3 and 4. Accordingly, a series of $N/100$ phthalate buffer solutions were prepared covering the pH range 2 to 6, and 1 ml. of an aqueous suspension of micro crystals of the globulin added to 100 ml. of each buffer solution, protected with toluene, and kept in the cold room until the electrophoresis experiments were completed.

We employed the method of Northrop and Kunitz (5), measuring under the microscope the migration velocity of the suspended crystal fragments and reversing the applied potential repeatedly. Measurements were made at both upper and lower stationary levels and the mean of a dozen or more closely agreeing observations for each pH were averaged.

The Helmholtz-Lamb equation $V = \zeta ND/4\pi h$ was used in calculating the electrokinetic potential. The values of D and h (dielectric constant and viscosity, respectively) have been assumed to be those for water at 25°C., namely, 81 and 0.009, respectively. All quantities in the above equation are expressed in c.g.s. electrostatic units.

The data are shown graphically in Fig. 1, from which it is clear that the isoelectric point of the tomato globulin is pH 3.43. Confirmation of this value for the isoelectric point was had by combining all of the

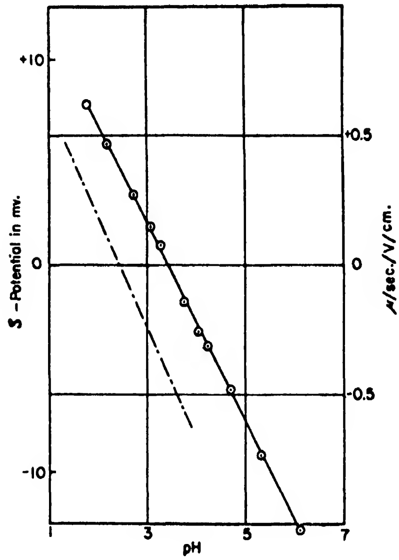

FIG. 1. Migration velocity and ζ-potential of tomato globulin.

various globulin solutions left over from the electrophoresis work and bringing the pH to 3.43, whereupon the protein separated out cleanly in a few minutes.

DISCUSSION

Cohn and coworkers (3) carried out some work on the electrophoresis of the red cellular debris of whole tomato juice and found zero migration around pH 4.69. It was of interest to repeat Cohn's work with the red cellular matter of juice sedimented in the Sharples centrifuge, after washing this several times with water and separating it by centrifuging. A small amount was suspended in phthalate buffer solutions of various pHs and the migration velocity, shown graphically as a broken line in Fig. 1, was obtained. Zero migration occurred at pH 2.40. It is evident that any protein adsorbed on the red cellular debris is not the globulin.

It follows that the globulin occurs in tomato juice (pH 4.2) as anion and can be removed by an anion exchange resin (IR-4) without going through the tedious dialysis operation. The new food accessory is left in solution and is removed subsequently by cation exchangers.

Acknowledgments

We are glad to acknowledge a grant from the Williams-Waterman Fund in partial support of the work, and also for supplies of exchange resin from the Resinous Products and Chemical Company.

Summary

The separation of a new crystalline globulin from tomatoes is described and its isoelectric point found to be pH 3.43.

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The Occurrence of Ergosterol in *Neurospora crassa*

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Introduction

Ergosterol, $C_{28}H_{44}O$, has been found to occur in molds thus far investigated, and hence may be considered the principal sterol of the lower fungi. In some instances (1), ergosterol has not actually been isolated, but its presence has been inferred because irradiation of the mycelium with ultraviolet light produced antirachitic activity.

Large quantities of *N. crassa* mycelia (strain 4540A) were accumulated in connection with the isolation and identification of a natural precursor of nicotinic acid (2). The mold was grown under carefully controlled conditions on Fries minimal medium (3) (2% sucrose) in 20-l. pyrex bottles at 30°C. for 4 days. The cultures were constantly aerated through a sterile filter. The mycelia were filtered off and dried in a forced-draft oven at 65°C. for 24 hr. and then ground in a Wiley mill. Yield of mycelia was approximately 80 g. (dry weight) from 20 l. of medium.

The identification of ergosterol is based upon the excellent agreement of the ultraviolet absorption spectrum of the sterol isolated from *Neurospora* with the spectrum of authentic ergosterol determined on the same instrument, as well as good agreement of the physical constants of the sterol, steryl acetate, and steryl benzoate with those of the corresponding derivatives prepared from authentic ergosterol. The sterol gives the typical color reactions (Liebermann-Burchard and Tortelli-Jaffé) of ergosterol.

Experimental

All melting points are corrected. All optical rotations were taken at room temperature in a 1.000 dm. tube, the sample being dissolved in 2.056 cc. of chloroform.

Isolation of Ergosterol

2610 g. of dry, finely ground mycelia were extracted with acetone in a Soxhlet apparatus for 110 hr. After removal of the acetone by distillation, the brown, oily residue weighed approximately 90 g. This was saponified with 400 g. of KOH in 2 l. of 70% ethanol; allowed to stand at room temperature for 72 hr., diluted to 4 l. with

water and extracted 6 times with 300 cc. portions of ether. The ether extracts were combined, washed 3 times with water, and dried for 24 hr. over anhydrous Na_2SO_4. The ether was distilled off in a N_2 atmosphere, and the crude sterol fraction, which weighed 4.8 g., was digested with methanol, crystallized, and dried. An orange color persisted, so the sterol was dissolved in ethanol and decolorized by the addition of a small amount of Norit. The recovered sterol weighed 3.22 g., m.p. 157–160°C. After 2 recrystallizations from ethyl acetate and 3 from ether in a Skau tube, small white plates were obtained, m.p. 160°C., $[\alpha]_D - 132°$. Ergosteryl acetate and ergosteryl benzoate were prepared by the usual methods. Acetate: m.p. 172°C., $[\alpha]_D - 89°$. Benzoate: m.p. 164°C., $[\alpha]_D - 64°$.

The ultraviolet absorption spectrum of the sterol (0.0599 mg./cc. in absolute ethanol) was determined on a Beckmann ultraviolet spectrophotometer. Peaks were found at 272 mμ, 282 mμ, and 294 mμ; this is in excellent agreement with the spectrum of authentic ergosterol determined on the same instrument. The spectra were found to be practically identical.

In subsequent determinations, the ergosterol concentration in *Neurospora crassa* was found, by quantitative digitonin precipitation, to be approximately 0.13% of the total dry weight of the mycelia.

Acknowledgments

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Summary

1. The principal sterol of *Neurospora crassa* has been isolated.

2. This sterol has been identified as ergosterol by means of the physical constants of the sterol, the steryl acetate, and the steryl benzoate; and also by means of the ultraviolet absorption spectrum.

3. The ergosterol concentration in *N. crassa*, grown in the manner described, has been found to be approximately 0.13% of the dry mycelial weight.

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The Influence of Growth and Adrenocorticotropic Hormones on the Fat Content of the Liver [1]

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Introduction

The increase in liver fat following injections of certain anterior pituitary extracts into fasting rats has been known for some time (1,2). It was not clear, however, which of the known factors in pituitary extracts is responsible for this effect. In fact, a separate ketogenic factor in the anterior pituitary has been suggested. It is the purpose of this paper to report the influence of acute dosage with pure growth and adrenoçorticotropic hormones on the liver fat content in fasting normal and hypophysectomized rats.

Experimental

Experiments in Normal Rats

Male rats of 40 days of age were fasted 24 hr.; on the second day of fasting, a total dose of 5.0 mg. of growth or adrenocorticotropic (ACTH) hormone, divided into 3 injections at 2-hr. intervals was administered intraperitoneally and the animals killed 2 hr. after the last injections. The livers were removed, weighed and placed into a dry-ice bath. The frozen tissues were dried *in vacuo*. The difference in weight before and after drying was taken as the water content. The dried powder was then extracted continuously by petroleum-ether in a Soxhlet apparatus. The ether extract was evaporated and the residue was dried to constant weight which was taken as the amount of total fat. The protein content in the fat-free dried tissue was estimated by multiplying Kjeldahl nitrogen by 6.25. The growth and adrenocorticotropic hormones were prepared by methods previously described (3,4).

It should be mentioned that the animals were fed *ad libitum* before fasting. The

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diet contained 19.3% protein, 56.7% carbohydrate, and 6.5% fat and was derived from 68.5% ground whole wheat, 5% casein, 10% alfalfa leaf meal, 5% fish oil, and 1.5% NaCl.

The results are summarized in Table I. It will be seen that the livers of rats receiving either the growth or adrenocorticotropic hormone have a higher fat content than do the livers of the controls. Adrenocorticotropic hormone appears to be somewhat more effective in this respect. The livers of the control animals contained 2.59% fat, whereas the amount of fat in the ACTH-treated livers was approixmately double this value (5.39%). On the other hand the liver fat of the growth-hormone injected rats was elavated from 2.59 to 4.27%. Statistical analysis of these differences showed them to be highly significant.

TABLE I

Effect of Growth and Adrenocorticotropic (ACTH) Hormones on the Liver Composition of Fasted Male Rats

Hormone injected[a]	No. of rats	Body weight at autopsy	Liver		Composition: G./100 g. liver		
			G.	G./100 g. b. wt.	Water	Fat	Protein
		g.					
Growth	8	154.1 ±9.1[b]	6.37	4.12 ±0.15	70.20 ±0.54	4.27 ±0.39	21.2 ±0.31
				(>0.7)[c]	(>0.3)	(0.001)	(>0.05)
ACTH	12	129.0 ±7.3	5.82	4.51 ±0.15	69.65 ±0.46	5.39 ±0.35	19.97 ±0.74
				(0.1)	(<0.1)	(<0.001)	(<0.05)
Control	20	133.5 ±5.1	7.67	4.13 ±0.15	70.97 ±0.47	2.59 ±0.21	21.66 ±0.33

[a] 40 days old male rats were fasted 24 hr.; on the second day of fasting, 3 injections with a total dose of 5.0 mg. growth hormone or ACTH were administered intraperitoneally to the animals in 2-hr. interval. Animals were killed 2 hr. after the last injection.

[b] Mean ± standard error.

[c] Fisher's p value.

It may be noted that the liver weight was not changed appreciably by the injections. The water content remained unchanged in spite of the increment of the liver fat. There appears to be a slight decrease in liver protein following the administration of adrenocorticotropic hormone.

Experiments in Hypophysectomized Rats

Similar experiments were carried out on male rats hypophysectomized at 40 days of age. Fasting began 6 days after operation. The results are shown in Table II. It will be observed that the animals

TABLE II

Effect of Growth and Adrenocorticotropic (ACTH) Hormones on the Liver Composition of Fasted Hypophysectomized Male Rats

Hormone injected[a]	No. of rats	Body weight at autopsy	Liver		Composition: G./100 g. liver		
			G.	G./100 g. b. wt.	Water	Fat	Protein
		g.					
Growth	10	121.0 ±4.8[b]	4.13	3.43 ±0.09 (<0.02)[c]	70.93 ±0.40 (<0.01)	3.38 ±0.25 (<0.001)	19.07 ±0.25 (<0.001)
ACTH	8	105.9 ±3.1	3.69	3.49 ±0.11 (<0.05)	69.02 ±0.27 (<0.02)	3.09 ±0.25 (0.001)	21.03 ±0.15 (<0.01)
Control	16	114.63 ±3.8	3.69	3.22 ±0.06	69.82 ±0.17	2.19 ±0.10	22.00 ±0.17

[a] Hypophysectomized male rats (operated at 40 days of age and 6 days postoperative) were fasted 24 hr.; on the second day of fasting, 3 injections with a total dose of 5.0 mg. growth or adrenocorticotropic hormone were administeree intraperitoneally to the animals in 2-hr. interval. Animals were killed 2 hr. after the last injections.

[b] Mean ± standard error.

[c] Fisher's *p* value.

receiving either growth or adrenocorticotropic hormone continued to show a definite increase in liver fat. The increase is about the same in both groups. While the liver weight per 100 g. body weight appears less in the hypophysectomized rats than that for unoperated animals there is no significant difference in the composition of protein and water. However, the fat content seems to be higher in the normal liver, a value of 2.59% *vs.* 2.19% for the hypophysectomized rats.

Discussion

The earlier experiments of Best and Campbell (1,2) established the fact that certain anterior pituitary extracts produce a rapid increase in size and an intense fatty infiltration of the liver in fasting rats. These observations have been confirmed subsequently by other investigators (5,6). It was further shown by MacKay and Barnes (5), and Fry (6), that the fatty liver normally produced by the administration of anterior pituitary extract does not occur following adrenalectomy. Thus, it may be said that the production of fatty liver is mediated by the adrenal cortex. The data herein reported using adrenocorticotropic hormone agree with this conclusion. In fact, Baker *et al.*, (7) have already shown that fatty infiltration occurs in adult rats treated with adrenocorticotropic hormone, as demonstrated by histochemical technique. The fact that adrenocorticotropic hormone directly or indirectly affects fat

metabolism has also been demonstrated by Bennett *et al.* (8). These investigators found that adrenocorticotropic hormone exhibits ketogenic activity in fasting rats and that the ketogenic effect of the hormone disappears in adrenalectomized animals.

The fact that growth hormone might cause an increase in liver fat could possibly have been expected. Earlier investigators working with only partially purified growth preparation (9,10) found growth-promoting and ketogenic activities were associated. The recent data of Bennett *et al.* (8) have clearly shown that pure growth hormone possesses ketogenic activity.

In unfasted normal or hypophysectomized rats treated with growth hormone for 10 days or more, we have found in each case a decrease of the fat content in the liver (11). The present experiment with fasting and acute dosage gives an opposite result. These data may be looked upon as indicating that the growth hormone causes at first a mobilization of depot fat to the liver, the fat being then either utilized for protein synthesis or oxidized as an energy source under further injections of the hormone.

Summary

The composition of the liver of fasting normal or hypophysectomized rats acutely treated with adrenocorticotropic or growth hormone has been analyzed. It has been shown that both growth and adrenocorticotropic hormone cause increase in liver fat with less significant changes in the water and protein content.

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The Enzymatic Synthesis of Glucose-1,6-Diphosphate

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Introduction

Evidence on the identity of the coenzyme of phosphoglucomutase (1) with glucose-1,6-diphosphate has been reported previously (2). This substance has been found to be synthesized by yeast under the same conditions which lead to an accumulation of fructose-1,6-diphosphate, and this was the starting point of this investigation in which the enzymatic synthesis of the coenzyme has been studied by incubating adenosinetriphosphate (ATP) with different sugar derivatives in the presence of muscle and yeast enzymes.

The coenzyme can be estimated fairly accurately by a method based on the acceleration of the phosphoglucomutase reaction: glucose-1-phosphate → glucose-6-phosphate. This is a very sensitive method by which amounts of the order of 10^{-10} moles can be measured, and it permits detection of the synthesis even with crude enzymes, in which case it is a very slow process in comparison with other competing reactions which transform the sugar derivatives.

The reaction mixture which gave by far the best yield of coenzyme was that of glucose-1-phosphate with ATP and the enzyme, and although the identity of the coenzyme was not known with certainty, at that time, the reaction was formulated provisionally as follows:

$$\text{glucose-1-phosphate} + \text{ATP} \rightarrow \text{glucose-1,6-diphosphate} + \text{ADP}. \quad \text{(I)}$$

With crude enzymes the yield was only about 0.5% of the glucose-1-phosphate added and this low yield was mainly due to the presence of phosphoglucomutase which disposed of most of the substrate.

The muscle enzyme could be purified sufficiently, so that, as much as 80% of the glucose-1-phosphate was transformed into coenzyme and it has been possible to test the validity of the previous equation.

According to this equation, one acid-labile phosphate group should become stable since the reactants contain 3 acid-labile groups and the reaction products only two. Moreover, the amount of glucose diphosphate should be equivalent to half of the acid-labile phosphate of ATP and one acid equivalent should be liberated per molecule of glucose diphosphate. These 3 conditions were found to agree with the experimental data.

The above mentioned reaction is similar to the hexokinase and phosphohexokinase reactions and it seems justified to call the enzyme: glucose-1-phosphate kinase. It has a pH optimum at 6.8, and it is activated by magnesium and manganese ions. Glucose and glucose-1-phosphate are phosphorylated in position 6 by hexokinase and glucose-1-phosphate kinase, respectively. The possibility that they were one and the same enzyme was investigated, and it was found that the two effects could be separated.

Methods

Substrates and Analytical Methods

Methods of estimation were as follows. Phosphoglucomutase and cophosphoglucomutase as described previously (2). Hexokinase by an adaptation of the method described by Trucco *et al.* (3) for galactokinase. Phosphate: Fiske and SubbaRow (4). Acid-labile phosphate after 7 min. hydrolysis in 1 N H_2SO_4 at 100°C. Protein: Kunitz and MacDonald (5). Glucose-6-phosphate was prepared from glucose-1-phosphate according to Colowick and Sutherland (6). Glucose-1-phosphate was obtained by chemical (7) or enzymatic (8) synthesis. Fructose-6-phosphate plus fructose-1-phosphate according to Macleod and Robison (9).

Estimation of the Enzyme

Unless otherwise stated, the reaction mixture for the determination of the enzyme was as follows: 0.5 μM of glucose-1-phosphate, 0.37 μM of ATP, 1 μM of $MgSO_4$; total volume, 0.1 ml. All solutions adjusted to pH 7.0. The reaction was started by adding variable amounts of enzyme. After 10 min. at 37°C., the reaction was interrupted by immersing the tubes in a boiling water bath. With the crude extracts it was often necessary to centrifuge and take an aliquot of the supernatant. Glucose diphosphate was then estimated by addition of glucose-1-phosphate and yeast phosphoglucomutase as described previously (2) using 70% pure glucose-1,6-diphosphate as standard. Blanks to measure any preformed coenzyme and reducing substances formed during the first reaction were run at the same time. These consisted of samples in which the glucose-1-phosphate kinase reaction was stopped at 0 time, and samples which were incubated but to which no phosphoglucomutase was added.

Criteria of Purification

The ratio of activities: glucose-1-phosphate kinase/phosphoglucomutase was generally used as a criterion of purification. As mentioned in the introduction, the low yield in glucose diphosphate with crude extracts was due to the competition of other enzymes for the same substrates. With the yeast extracts, the activity of phosphoglucomutase explained almost quantitatively the lack of recovery, and, for this reason, the above-mentioned ratio was used to measure the purification.

Another criterion was the maximum yield of coenzyme relative to the glucose-1-phosphate added. It was measured in the same mixture as that used for the estimation of activity but with an excess of ATP (0.7 μM). Aliquots were withdrawn from the mixture every 30 min., and the glucose diphosphate formed was measured until a maximum was reached.

Experimental

Specificity of Glucose-1-Phosphate

Several sugars and hexose phosphates were incubated with Lebedew's juice and an excess of ATP. Glucose, fructose and their monophosphates increased the formation of coenzyme. Glucose-1-phosphate seemed to be the best substrate, but the results were not conclusive. However, when the maceration juice was fractionated with $(NH_4)_2SO_4$ and the fraction which precipitates at 45% saturation tested, it became clear that glucose-1-phosphate gave the largest yield of coenzyme. When muscle enzyme was purified no detectable amount of coenzyme was synthesized with any substrate except glucose-1-phosphate (Table I).

TABLE I

Coenzyme Formation from Different Substrates

1 μM of substrate, 0.5 μM of ATP, 2.0 μM $MgSO_4$, and 0.005 ml. of yeast enzyme, or 0.01 ml. of muscle enzyme, were incubated for 10 min. at 37°C. Total volume, 0.5 ml. Results in moles 10^{-10}.

Substrate	Cophosphoglucomutase formed	
	Yeast enzyme	Muscle enzyme
None	0.0	0.0
Glucose-1-phosphate (no ATP)	0.05	0.0
Glucose-1-phosphate	4.00	>8.0
Glucose	1.83	—
Fructose	1.03	—
Glucose-6-phosphate	0.38	0.0
Glucose-6-phosphate+glucose-1-phosphate	4.08	>8.0
Fructose-1-phosphate and fructose-6-phosphate	0.22	—
Fructose-1-phosphate and fructose-6-phosphate+glucose-1-phosphate	3.03	—
Glucose-6-phosphate+fructose-6-phosphate	—	0.0

Purification of Yeast Enzyme

A maceration juice of dried brewers' yeast (*Cerveceria palermo*) was prepared according to Neuberg and Lustig (10). To 400 ml. of this juice 1 N acetic acid was added until pH 4.9 was reached. The small isoelectric precipitate was centrifuged at 6000 r.p.m. and the supernatant discarded. The precipitate was dissolved in 20 ml. of water, and the pH raised to 6.8. The solution was then precipitated by adding 0.9 volumes of saturated $(NH_4)_2SO_4$ and the precipitate dissolved in 1–2 ml. of water. By this procedure the relation phosphoglucokinase/phosphoglucomutase was increased about 40 times, and the recovery of glucose-1-phosphate as coenzyme increased from less than 0.5% in the maceration juice to 12–17% in the purified solution (Table II). Unfortunately, the loss of enzyme was so great (about 95%) that further attempts of purification of the yeast enzyme were discontinued.

TABLE II

Purification of Yeasts' Phosphoglucokinase

Results in moles $\times 10^{-9}$ of reaction product.

	Coenzyme formed	Glucose-6-phosphate formed	Maximum. Per cent glucose-1-phosphate recovered as coenzyme
Lebedew juice	1.2	150.0	~0.5
Isoelectric precipitation	2.2	40.0	—
Ammonium sulphate precipitate	3.0	10.0	12.0

Purification of Rabbit Muscle Enzyme

Rabbits were killed by a blow and bled. The muscle was passed through a cooled mincer and suspended with 2 volumes of cold water. After 10 min. extraction it was strained through muslin and extracted again with 1 volume of water. A third extraction was made with 1 volume of salt solution containing 0.5 M KCl and 0.1 M phosphate buffer pH 7.4. This procedure was carried out with 14 rabbits. In 4 of them the first water extract was the richest in phosphoglucokinase (highest value 1.2×10^{-3} μM of glucose diphosphate synthesized by 0.01 ml. in 10 min.); and in 5 cases the saline extract was the richest (highest value 2.2×10^{-3} μM of glucose diphosphate synthesized by 0.01 ml. in 10 min.); finally, in 3 cases, no appreciable activity was obtained in any of the 3 extracts. In every case the saline extract was chosen for further purification, because even in those instances in which the activities were poorer than in the aqueous extracts, the relation kinase/mutase was always highest.

In the saline extract the phosphoglucokinase behaves as if it were loosely adsorbed on myosin. When this solution was dialyzed as a thin layer against distilled water, protein started to precipitate after about 1 hr., and finished in about 4 hr. This precipitate, which was mainly

myosin, carried down the phosphoglucokinase activity. On the contrary, when myosin was precipitated with $(NH_4)_2SO_4$ at 33% saturation, practically no activity followed the precipitate. Fifty per cent saturated $(NH_4)_2SO_4$ is necessary to precipitate the activity (Table III).

TABLE III

Precipitation of the Muscle Enzyme by Dialysis and Ammonium Sulphate Fractionation
Results in moles $\times 10^{-10}$.

Treatment	Glucose diphosphate formed		Protein/mg./ml.	
	Supernatant	Precipitate	Supernatant	Precipitate[a]
Dialysis				
2 hr.	1.2	2.0	—	—
4 hr.	0	3.8	1	0.73
Ammonium sulphate treatment				
33% saturation	—	0.7	—	1.1
50% saturation	—	>8.0	—	0.9

[a] Dissolved in 0.05 *M* phosphate buffer pH 7.4.

Precipitation with $(NH_4)_2SO_4$ was the best way of purifying the saline extracts. To 110 ml. of this extract, 80 ml. of saturated $(NH_4)_2SO_4$ solution, brought to pH 7.5 with NH_4OH, was added. The precipitate was separated by filtration through fluted paper in the ice-box. One hundred fifty ml. of clear filtrate were obtained, to which 25 ml. of $(NH_4)_2SO_4$ (pH: 7.5) were added. The precipitate was now collected by filtration and dissolved in water to make 8 ml. of solution.

As shown in Table IV, this procedure improves the relation kinase/mutase about 40 times, and the relation kinase/protein 4-fold. The liberation of inorganic phosphate effected by the crude enzyme was not detectable with the purified solution.

Relation between Hexokinase and Glucose-1-Phosphate Kinase Activities

The possibility that the hexokinase and the glucose-1-phosphate kinase reactions were catalyzed by the same enzyme was investigated by measuring both activities in different extracts.

TABLE IV

Activities of the Muscle Enzymes during Purification

Tests as described in text, referred to 0.01 ml. enzyme. Results in moles $\times 10^{-9}$ of reaction product.

	Coenzyme formed	Glucose-6-phosphate formed	Inorganic phosphate liberated	Protein mg./ml.
Aqueous extract (rejected)	0.24	12.0	—	—
Crude saline extract	0.6	8.0	2.0	8.0
Fraction insoluble in 0.33 saturated ammonium sulphate	0.35	2.0	3.0	—
Fraction precipitated in 0.5 saturated ammonium sulphate	3.6	1.2	0	10.5

These two activities were measured in: (a) Crude Lebedew juice; (b) A solution of hexokinase obtained from brewers' yeast by the method of Berger *et al.* (11) and partially purified by acetone precipitation; and (c) The partially purified preparations of glucose-1-phosphate kinase.

The results are shown in Table V. The purified hexokinase had the lowest glucose-1-phosphate kinase activity. On the other hand, the ratio hexokinase/glucose-1-phosphate kinase was about 30 times lower in the purified glucose-1-phosphate kinase than in Lebedew juice.

The two activities were thus found to change independently from the point at which it was concluded that different enzymes were involved in these reactions.

TABLE V

Hexokinase and Glucose-1-Phosphate Kinase Activities in Different Enzyme Preparations

Values referred to 0.01 ml. enzyme. Results in moles $\times 10^{-9}$ of reaction product.

Preparation	Hexokinase	Glucose-1-phosphate kinase
Lebedew juice	10200	0.7
Purified glucose-1-phosphate kinase from yeast	1240	2.4
Partially purified hexokinase	8000	0.08
Purified glucose-1-phosphate kinase from muscle	0	3.7

Yield of Coenzyme in Relation to the Amount of Adenosinetriphosphate

Known amounts of ATP were incubated at 37°C. with glucose-1-phosphate, magnesium and the enzyme. Samples were withdrawn at various times, and the reaction stopped by heating. The amount of coenzyme was then estimated at suitable dilutions as usual. The enzyme used was a purified preparation from muscle in which the $(NH_4)_2SO_4$ fractionation had been repeated twice and which was practically free from adenosinetriphosphate and phosphatase activity.

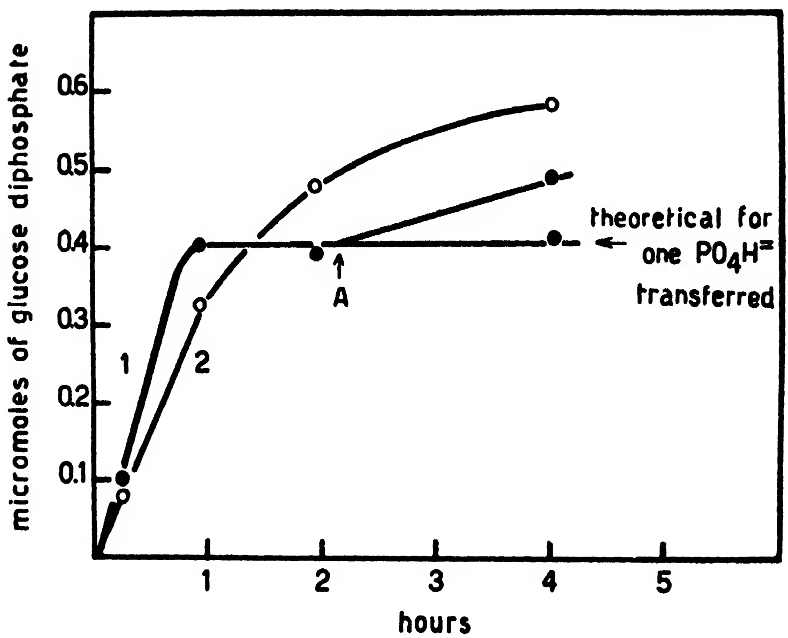

FIG. 1. Yield of coenzyme in relation to the amount of ATP. Curve 1. Incubation of 0.8 μM glucose-1-phosphate + 1.5 μM magnesium + 0.4 μM ATP + 0.01 ml. of enzyme. At point A, 0.2 μM ATP added to an aliquot. Curve 2. Same as 1 but with 0.8 μM of ATP.

The results of one of several experiments appear in Fig. 1, where Curve 1 shows that the maximum amount of coenzyme formed is equal to the amount of ATP, in agreement with Eq. 1. That the reaction stopped due to the lack of ATP was proved by adding more ATP to an aliquot, and finding that more coenzyme was synthesized.

Curve 2 in Fig. 1 shows the same type of experiment, but with double the amount of ATP. Here the end of the reaction was not attained, and the reaction seemed to take place more slowly. This inhibition by higher concentration of ATP has been repeatedly observed.

Yield of Coenzyme in Relation to the Decrease in Acid-Labile Phosphate

According to Eq. 1, one mole of acid-labile phosphate should become acid-stable per mole of coenzyme formed. Experiments were carried out in which the acid-labile phosphate and coenzyme concentration were estimated during the course of the reaction.

As shown in Fig. 2, the results agree with the proposed equation.

Yield of Coenzyme in Relation to Acid Production

Colowick and Kalckar (12) showed that, in the hexokinase reaction, one acid equivalent is liberated for every mole of phosphate trans-

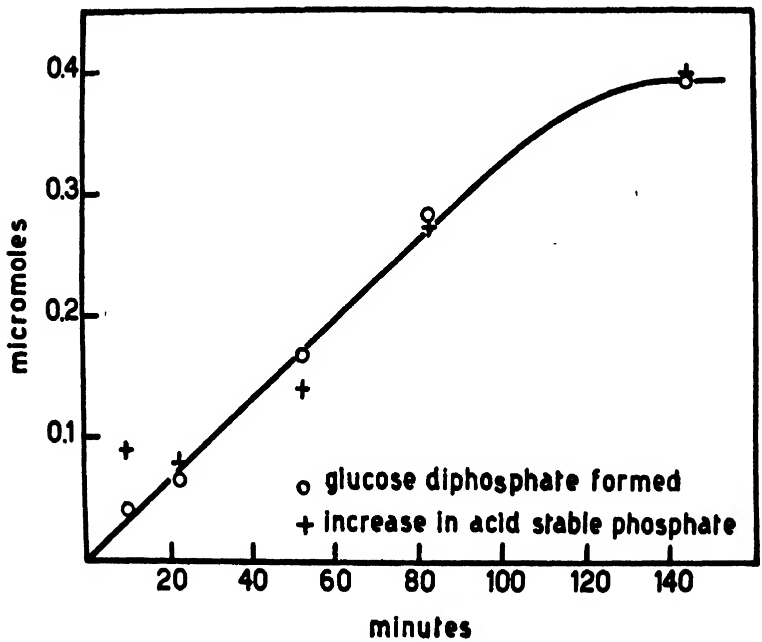

FIG. 2. Yield of coenzyme in relation to the increase in acid-stable phosphate. Incubation of 0.86 μM glucose-1-phosphate+0.56 μM ATP+1.5 μM $MgSO_4$+0.01 ml. of purified muscle enzyme. Reaction stopped by heating.

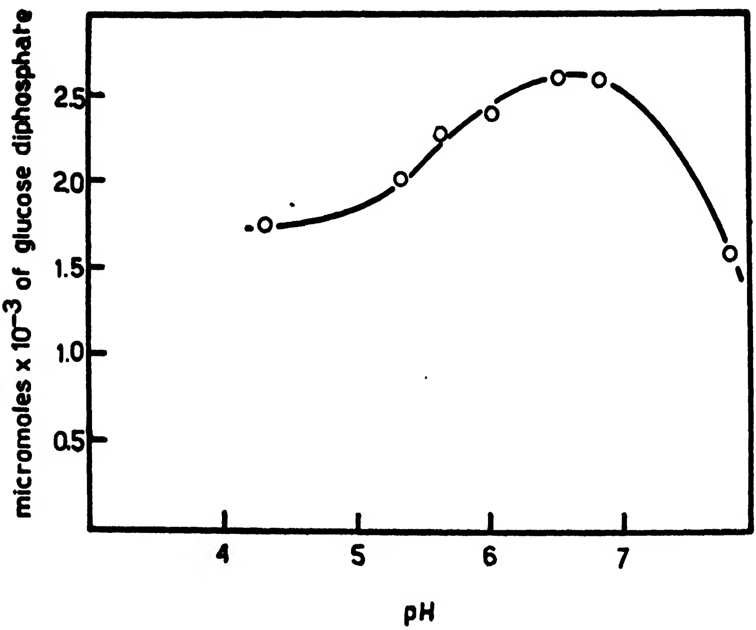

FIG. 3.

ferred from ATP to glucose. In the similar equation proposed for the synthesis of cophosphoglucomutase one acid equivalent should be produced for every mole of coenzyme formed.

The acid production was measured manometrically in a Warburg apparatus. In the main compartment were added: 10 μM of glucose-1-phosphate; 5 μM of $MgSO_4$; 3.7 μM ATP; 0.2 ml. of 0.5 M bicarbonate, and 0.1 ml. toluene. In the side bulb 0.2 ml. of purified muscle glucose-1-phosphate kinase. Total volume, 2.5 ml. Gas phase 95% N + 5% CO_2. A control without glucose-1-phosphate was run at the same time. After 35 min. the reaction was finished as judged by the pressure, which remained constant for 15 min. The CO_2 evolved was 77 μl corresponding to 3.5 microequivalents of acid. The enzymatic determination gave 3.8 μM of coenzyme.

Optimum pH Curve

Maleate "buffer" according to Smits (13) was used after it was ascertained that it does not interfere with the activity of the enzyme. The experiment of Fig. 3 was carried out with the muscle enzyme, and

shows that the optimum is at pH 6.8. With the yeast enzyme a closely similar curve was obtained.

Activation by Magnesium and Manganese

Fig. 4 shows that both Mg^{++} and Mn^{++} activate glucose-1-phosphate kinase. With magnesium the enzyme is fully activated at about 1.5×10^{-3} mole/l. Manganese is slightly less effective.

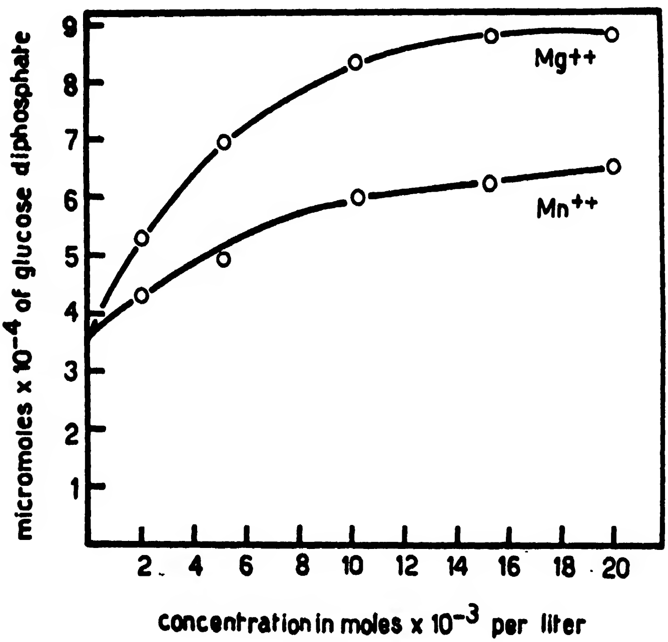

FIG. 4.

Since the variable concentration of ions might affect the activity of phosphoglucomutase during the estimation of glucose diphosphate, in these experiments, the amount of salt was made the same in all the tubes, including the coenzyme standard, before adding the phosphoglucomutase.

DISCUSSION

The evidence presented seems sufficient to show that in yeast and animal tissues glucose diphosphate is formed by the interaction of adenosinetriphosphate with glucose-1-phosphate. This raises the

question as to whether this reaction can be a quantitatively important pathway in carbohydrate dissimilation, or if it is only limited to the formation of coenzyme for phosphoglucomutase. The evidence at hand favors the second alternative. In crude extracts of yeast, the reaction catalyzed by hexokinase is several thousand times faster than that of glucose-1-phosphate kinase, and in either muscle or yeast the relation phosphoglucomutase/glucose-1-phosphate kinase is about 100, even under conditions devised to favor the activity of glucose-1-phosphate kinase.

The possibility remains that the glucose diphosphate is destroyed almost as fast as it is formed, but preliminary experiments indicate that this is not the case with muscle. However, the question is not definitely settled since extracts of liver, kidney, brain, and *Escherichia coli* destroy glucose diphosphate.

Another question which arises is whether the reaction of glucose-1-phosphate and ATP is the only mechanism by which glucose diphosphate is formed. Experiments indicate that *E. coli* can synthesize glucose diphosphate by the transference of phosphate between two molecules of glucose-1-phosphate without the intervention of ATP.

Summary

The enzymatic syntheses of glucose diphosphate have been studied with extracts of yeast and rabbit muscle. The best yields were obtained on incubation of the enzyme with adenosinetriphosphate and glucose-1-phosphate.

Using partially purified extracts for the estimation of acid-labile phosphate, the total yield of glucose diphosphate and the acid formation agree with the equation:

$$\text{ATP} + \text{glucose-1-phosphate} \rightarrow \text{glucose diphosphate} + \text{ADP}.$$

Magnesium and manganese ions accelerate the reaction. The optimum pH is 6.8.

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Studies on the Mechanism of the Inhibition of Glucolysis by Glyceraldehyde [1]

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Introduction

Twenty years ago, Mendel (1) discovered the inhibitory effect of glyceraldehyde on glucolysis. He found that 10^{-3} *M* *dl*-glyderaldehyde inhibited the anaerobic glucolysis of tumor cells almost completely without affecting their respiration or the respiration of normal cells. Later, Mendel, Bauch and Strelitz (2) showed that the inhibitory effect of glyceraldehyde is completely abolished by 10^{-3} *M* pyruvic acid and partially reversed by smaller amounts of this compound. These findings have been confirmed and extended to other tissues (9).

Studies by Adler (3) and Süllmann (4,5) suggested that glyceraldehyde interfered with the initial phosphorylation of glucose, a conclusion reached on the basis of the absence of any inhibitory effect of glyceraldehyde on the glycolysis of hexose mono- and diphosphates.

Neither Holmes (6) nor Lehmann (7) could obtain an inhibition by glyceraldehyde of muscle glycolysis in extracts to which yeast hexokinase was added. Adler *et al.* (3) could obtain no inhibition of yeast hexokinase activity with the above compound. Stickland (8), on the other hand, found that the glucolysis of muscle extracts fortified with yeast hexokinase was inhibited by 3×10^{-3} *M* glyceraldehyde, and that the inhibition could be reversed by gradually increasing the concentration of hexokinase. Thus, the amount of hexokinase present seemed to be the critical factor in determining the extent of the inhibition, and the aforementioned negative results were explained on this basis. Stickland also found that pyruvic acid (4×10^{-4} *M*) could reverse the effect of glyceraldehyde. These results suggested that the inhibition of hexokinase activity was the means whereby glucolysis was inhibited by glyceraldehyde.

Dorfman (9) has adequately reviewed and discussed the early literature on the

[1] This research was supported by a grant from the Ontario Cancer Treatment and Research Foundation.

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inhibitory action of glyceraldehyde on glycolysis, and points out that several questions regarding the mechanism of the effect remain unanswered: (1) How does pyruvic acid reverse the inhibition? (2) If glyceraldehyde inhibits glucolysis, why are the same concentrations not effective in inhibiting respiration? This last point has been raised by Barker *et al.* (10) in the case of the lack of inhibition of respiration by iodoacetic acid in concentrations which inhibit glycolsyis.

In view of these questions, it was decided to reexamine the problem of the mechanism of the inhibition of glucolysis by glyceraldehyde, and the action of pyruvate thereon, by studying the effect of these substances on the hexokinase of brain, muscle, tumor, and yeast.

Experimental

Methods

The method of Colowick *et al.* (11), whereby glucose disappearance is determined as a measure of hexokinase activity, was used. It is based on Nelson's colorimetric micro method (12) involving copper reduction and the use of $Ba(OH)_2$ and $ZnSO_4$ as protein precipitants, and has the desirable feature of eliminating all phosphorylated compounds during the protein precipitation.

In the experiments to be reported below, the procedure described by Colowick *et al.* was followed closely, and the concentrations of the components of the test system were exactly as given by them, unless otherwise indicated. The final volume of the incubation mixture was 2.35 ml. and the incubation period was 20 min. at 30°C. Glyceraldehyde and pyruvate were always placed in the main compartment of the Warburg vessel. After deproteinization, glucose was determined according to the method of Somogyi (13), using Nelson's arsenomolybdate reagent. Measurements of optical density were made in a Colemen Junior Spectrophotometer at 550 mμ.

The differences in reduction obtained before and after the experimental period were taken as reflecting the change in concentration of glucose. Errors due to the disappearance of small amounts of glyceraldehyde during the course of the experiment were corrected by heating an aliquot of the deproteinized filtrate for 4 min. at 80°C., and then immediately cooling in ice water. Under these conditions most of the reduction due to glyceraldehyde had already taken place, while that due to glucose was just beginning. From the difference in optical density readings obtained between controls and experimental reaction mixtures at the above time and temperature, it was possible to determine the amounts of glyceraldehyde disappearing with an error of $\pm$ 10%. The difference in density readings obtained from the reaction mixtures treated as above was then subtracted from the difference in densities found after complete reduction by glyceraldehyde and glucose had taken place in another aliquot of the same filtrate. Using this method of correcting for the disappearance of glyceraldehyde, it was found possible to determine with an accuracy of $\pm$ 5–8% the amount of glucose disappearing during an interval when *small* amounts of glyceraldehyde also disappeared. This method was considered sufficiently accurate for the purpose of determining whether a marked inhibition of hexokinase activity existed. In the experimental results tabulated below, differences in inhibition of less than 15% are not

considered significant. Experiments were usually arranged so that the differences in optical density readings in the absence of glyceraldehyde after heating for 10 min. at 100°C. were very large as compared with those obtained after heating for 4 min. at 80°C. All values listed in the tables below are corrected for glyceraldehyde disappearance.

Materials

The glyceraldehyde used was the *dl*-form prepared by Schering-Kahlbaum as a white powder. The latter was washed by refluxing with acetone for 30 min, filtered, and then dried. (M.P. 138°C.) Solutions used for enzymatic experiments were heated for 5 min. at 85°C. to ensure only the monomeric form being present. Needham and Lehmann (7) have shown that the dimeric form does not inhibit glucolysis. Pyruvic acid was added in the form of the sodium salt prepared according to Robertson (14). Adenosine triphosphate was prepared by the method of Kerr (15). Samples from commercial sources were also used.[3]

The hexokinase extracts from beef brain acetone powders and from rat skeletal muscle were prepared as described by Colowick *et al.* (11), the muscle extract being obtained by filtration through cheese cloth. Tumor hexokinase was prepared in two ways, as a homogenate and as an aqueous extract from an acetone powder of rat sarcoma 39. The former was made by homogenizing 1 g. of tumor tissue freed from necrotic areas with 1.5 ml. of distilled water at 5°C., while the latter preparation was made in the same manner as the brain acetone powder extract. The hexokinase from yeast was prepared according to the method of Meyerhof (16).

Results

The Effect of Glyceraldehyde and Pyruvate on the Hexokinase Activity of Beef Brain Extracts

Experiments were performed to determine whether glyceraldehyde exerted an inhibitory effect on the activity of hexokinase from beef brain extract and the effect of pyruvic acid thereon. Table I shows the results of these experiments. From this table it will be seen that glyceraldehyde in concentrations varying from 2×10^{-3} *M* to 4.5×10^{-3} *M* exerts an inhibitory effect on hexokinase activity ranging from 44 to 100%. These results support the assumption of earlier workers that glyceraldehyde acts by inhibiting the phosphorylation of glucose.

The effect of pyruvate on the inhibitory action of glyceraldehyde on beef brain hexokinase is somewhat uncertain. In one case the inhibition was partially reversed (Expt. 5, Table I), while in the majority of cases no significant effect could be noted. Control experiments show that glyceraldehyde did not react with pyruvate in the presence of the

[3] Armour and Company, Chicago, Ill., and Nutritional Biochemicals, Cleveland, Ohio.

TABLE I

The Effect of Glyceraldehyde and Pyruvate on the Activity of the Hexokinase from Beef Brain Cortex

Experiment no.	Molar conc. of glyceraldehyde	Molar conc. of pyruvate	Normal γ of glucose disappearing	Per cent inhibition		
				With glyceraldehyde	With pyruvate + glyceraldehyde	With pyruvate
1	4.5×10^{-3}		450	100		
2	4.5×10^{-3}		505	86		
3	2.2×10^{-3}		465	44		
4		1×10^{-4}	220			0
5	3.5×10^{-3}	1×10^{-3}	530	52	24	
6	4.5×10^{-3}	5×10^{-4}	400	82	66	
7	4.0×10^{-3}	2×10^{-3}	375	64	52	
8	2.2×10^{-3}	5×10^{-4}	500	54	56	
[a]9	4.5×10^{-3}	2×10^{-3}	410	53	48	
[a]10	4.5×10^{-3}	2×10^{-3}	580	72	50	
[b]11	4.5×10^{-3}		630	14		
[b]12	4.5×10^{-3}		485	17		
13	4.5×10^{-3}	4×10^{-3}	325	100	100	
14	4.5×10^{-3}	8×10^{-3}	325	100	100	

0.6 ml. of brain extract were added to the main compartment of the Warburg cup containing 1.0 ml. of 0.02 *M* $MgCl_2$, 0.06 *M* $NaHCO_3$, with water, glyceraldehyde, and pyruvate to a final volume of 1.9 ml. The sidearm contained 0.1 ml. 1% glucose, 0.15 ml. 0.9 *M* NaF, and 0.05 *M* $NaHCO_3$, and 0.2 ml. of 0.05 *M* adenosine triphosphate. Final volume 2.35 ml. Incubation period: 20 min. at 30°C.

[a] Extract incubated for 20 min. at 20°C. with glyceraldehyde before reaction initiated.

[b] *d*-Glyceraldehyde used in these experiments. In all other cases the racemic form was employed.

extract, nor did pyruvate display any reducing activity on the copper reagent. The lack of reaction of pyruvate with glyceraldehyde was found to be true for all other extracts from different sources mentioned forthwith.

Tests with slices of beef brain cortex showed that the amounts of glyceraldehyde necessary to inhibit glucolysis of this tissue are much lower than those normally required to cause the same degree of inhibition of the hexokinase in such preparations. For example, 10^{-3} *M* glyceraldehyde, which has no effect on the activity of the extract, inhibits the anaerobic glucolysis of slices 80-90%, irrespective of the

temperature at which the estimation was carried out (37.5°C. or 30°C.). Meyerhof and Randall (17) obtained similar results in their studies on the inhibitory effect of adrenochrome on glycolysis. There may be several reasons for this phenomenon, which will be discussed further on in this paper. No effect of pyruvate could be noted on the inhibition of glucolysis in beef brain slices effected by glyceraldehyde. This fact harmonizes with the findings obtained with extracts.

Needham (18) and Mendel (19) found that the *l* isomer of glyceraldehyde was responsible for the inhibition of gucolysis. To ascertain which enantiomer was exerting the inhibitory effect in the investigation reported here experiments were carried out with *d*-glyceraldehyde.[4] Expts. 11 and 12, Table I, show that *d*-glyceraldehyde has a negligible effect on the activity of the hexokinase. It can be concluded, therefore, that the *l* form only is responsible for the inhibition observed with a racemic mixture. It was noted, furthermore, that, during the course of the experiments, small amounts of *d*-glyceraldehyde disappeared, which were comparable to those amounts disappearing in experiments with *dl*-glyceraldehyde. It is, therefore, very probable that the glyceraldehyde being metabolized in the experiments where the racemic form was employed was of the *d* form.

The Effect of Glyceraldehyde on Tumor Hexokinase Activity

The effects of glyceraldehyde and pyruvate were determined on homogenates and on aqueous extracts of acetone powders of rat sarcoma 39. The results are shown in Table II. From this table it will be seen that 4.5×10^{-3} glyceraldehyde is needed to bring about a 50–60% inhibition of the purified hexokinase extracts, while the homogenates show only a 30% inhibition when the same concentration is used. The greater sensitivity of the extracts could be due to the lack of protective substances present in the homogenates. In this connection, it is interesting to note that Boyland (21) found that, with glycolizing tumor extracts, a 90% inhibition could be achieved only with 2.2×10^{-2} *M* glyceraldehyde. Pyruvate does not significantly affect the inhibition by glyceraldehyde in either type of preparation. The presence or absence of fluoride does not seem to influence the lack of effect of pyruvate (Expts. 9 and 10, Table II).

[4] The author is indebted to Dr. E. Baer for his kindness in supplying a sample of *d*-glyceraldehyde used in this investigation.

TABLE II

The Effect of Glyceraldehyde and Pyruvate on the Activity of Hexokinase from Rat Tumor

Experiment no.	Molar conc. of glyceraldehyde	Molar conc. of pyruvate	Normal γ of glucose disappearing	Per cent inhibition		
				With glyceraldehyde	With pyruvate + glyceraldehyde	With pyruvate
1	1.0×10^{-3}	2.0×10^{-3}	480	26	9	0
2	1.5×10^{-3}	2.0×10^{-3}	370	31	32	0
3	3.0×10^{-3}	4.0×10^{-3}	515	12	12	0
4	4.5×10^{-3}	4.0×10^{-3}	470	25	16	0
5	4.5×10^{-3}	4.0×10^{-3}	400	26	26	
6	4.5×10^{-3}	4.0×10^{-3}	500	58	58	
7	4.5×10^{-3}	4.0×10^{-3}	470	55	58	0
8	4.5×10^{-3}	4.0×10^{-3}	690	61	71	
9	4.5×10^{-3}	4.5×10^{-3}	890	45	45	
10	4.5×10^{-3}	4.5×10^{-3}	860	48	48	0

In Expts. 1 to 5, 0.4 ml. of homogenate was used. In all other experiments, 0.4 ml. of an aqueous extract of acetone powder was used. In Expts. 9 and 10, no fluoride was present. Reaction time: 20 min. at 30°C. in all cases. Other conditions as described in footnote to Table I.

When the action of glyceraldehyde and pyruvate was tested on the anaerobic glucolysis of tumor tissue slices, profound differences from the behavior of cell-free extracts were noted. The sensitivity of slices to small concentrations of glyceraldehyde was much greater than that observed with homogenates. Moreover, pyruvate definitely reversed the inhibition, the extent of the reversal varying according to the concentration of glyceraldehyde used. Typical experiments are outlined in Table III. It was also found that, when the disappearance of glucoes was used as the criterion for the extent of glucolysis, similar results were obtained (Table IV). It is enteresting to note that in the second experiment of Table IV, 10^{-4} M pyruvate was sufficient to abolish a 59% inhibition by 10^{-3} M glyceraldehyde. In later experiments with tissue slices, where glucose disappearance was measured, it was also found that about 50% of the glyceraldehyde present had disappeared at the end of the experimental period, both in the absence and in the presence of pyruvic acid. It was not possible to asceratin with any

great accuracy the amounts disappearing and it was difficult to determine whether only *d*-glyceraldehyde disappeared or the *l* form also. If more of the *l* form disappears in the presence of pyruvic acid, it would readily explain how the reversal of the inhibition is affected.

TABLE III

The Effect of Glyceraldehyde and Pyruvate on the Glucolysis of Slices of Rat Sarcoma 39

Additions to normal medium	$Q_{CO_2}^{N_2}$	Inhibition *per cent*
(1) None	30.0	—
10^{-3} *M* pyruvate	34.5	—
1.5×10^{-3} *M* glyceraldehyde	5.0	84.0
1.5×10^{-3} *M* glyceraldehyde + 10^{-3} *M* pyruvate	29.5	0.0
10^{-3} *M* glyceraldehyde	7.0	77.0
10^{-3} *M* glyceraldehyde + 10^{-3} *M* pyruvate	33.5	0.0
5×10^{-4} *M* glyceraldehyde	27.0	10.0
5×10^{-4} *M* glyceraldehyde + 10^{-3} *M* pyruvate	35.5	0.0
(2) None	20.0	—
4.0×10^{-3} *M* pyruvate	22.5	—
4.5×10^{-3} *M* glyceraldehyde	4.0	80.0
4.5×10^{-3} *M* glyceraldehyde + 4×10^{-3} *M* pyruvate	11.0	45.0

Glucolysis was measured at 37.5°C. in section (1) and at 30°C. in section (2). The medium consisted of Ringer's bicarbonate solution containing 200 mg.-% glucose. The gas phase was 95% N_2–5% CO_2.

$Q_{CO_2}^{N_2}$ = μl. CO_2/hr./mg. dry weight, calculated on the basis of pressures obtained during the first 30 min. period after temperature equilibration.

TABLE IV

The Effect of Glyceraldehyde (GA) and Pyruvate (PY) on the Disappearance of Glucose Effected by Slices of Rat Sarcoma 39

	γ of glucose disappearing/mg. of tissue (dry weight) in 2.5 hr.		
	I	II	III
Normal	128	113	96
Normal + 10^{-3} *M* Py	145	115	98
Normal + 10^{-3} *M* Py + 10^{-3} *M* GA	90	110	83
Normal + 10^{-4} *M* Py + 10^{-3} *M* GA	45	90	45
Normal + 10^{-3} *M* GA	48	56	52

The medium used was the same as that used for the experiments with tissue extracts except that no fluoride or adenosine triphosphate were present.

The Effect of Glyceraldehyde on Yeast Hexokinase

The measurements of yeast hexokinase activity are the most accurate of all those reported in this paper, since it was found that no glyceraldehyde disappeared during the course of the reaction. The preparation obtained according to the procedure of Meyerhof had a dry weight of 55 mg./ml. 0.15 ml. of the 10-fold diluted preparation, corresponding to 0.83 mg. of protein, was used in the experiments. A similar preparation of yeast hexokinase, used by Stickland (8), had a dry weight of 18 mg./ml. and, since he added 0.2 ml. of extract (corresponding to 3.6 mg. of protein), the amount of yeast hexokinase used in the following experiments was much less than that used by Stickland. Despite this fact, the inhibition of yeast hexokinase requires extremely large amounts of glyceraldehyde (Table V). Concentrations which would inhibit muscle hexokinase 80–100% have a comparatively mild effect on the yeast hexokinase. Under the conditions of Stickland's experiments, where yeast hexokinase was added to a muscle extract, 3×10^{-3} *M* glyceraldehyde almost completely inhibited glucolysis. Pyruvic acid

TABLE V

The Effect of Glyceraldehyde and Pyruvate on the Activity of the Hexokinase from Yeast

Experiment no.	Molar conc. of glyceraldehyde	Molar conc. of pyruvate	Normal γ of glucose disappearing	Per cent inhibition	
				With glyceraldehyde	With pyruvate + glyceraldehyde
1	1.0×10^{-3}		550	5	
2	1.0×10^{-3}		475	20	
3	3.0×10^{-3}	4.0×10^{-3}	495	13	9
4	3.0×10^{-3}	3.0×10^{-3}	610	56	63
5	3.0×10^{-3}	3.0×10^{-3}	655	30	26
6	6.0×10^{-3}	6.0×10^{-3}	480	48	48
7	6.0×10^{-3}	6.0×10^{-3}	460	59	0
8	6.0×10^{-3}	6.0×10^{-3}	380	59	59
9	6.0×10^{-3}		515	4	
10	1.2×10^{-2}		680	50	

0.15 ml. of the 10-fold diluted extract was used as the source of enzyme. No fluoride was present in any of the above experiments. Other conditions as described in footnote to Table I.

was found to be completely without effect on the inhibitory action of glyceraldehyde, except for one instance (Expt. 7, Table V). In no case could this experiment be repeated, however. The foregoing results with pyruvate appear to be directly at variance with those of Stickland, who found that 4×10^{-4} *M* pyruvate could reverse completely the inhibition of glucolysis caused by 3×10^{-3} *M* glyceraldehyde. However, it will be recalled that Stickland was measuring glucolysis presumably in the presence of an intact series of glycolytic enzymes from muscle. Thus, the action of glyceraldehyde on glycolizing cell-free tissue extracts may present features which are similar to those observed in the present study with tumor slices, where it has been shown that the glyceraldehyde inhibition of the glucolysis of tumor slices can be reversed by small amounts of pyruvic acid.

The Effect of Glyceraldehyde on Muscle Hexokinase Activity

The effect of various amounts of glyceraldehyde and pyruvic acid on the hexokinase activity of rat skeletal muscle extracts was examined and the results are shown in Table VI. From these results it can be seen the 2×10^{-3} *M* glyceraldehyde inhibits muscle hexokinase activity 80–100%. Thus, under the conditions adopted in these experiments

TABLE VI

The Effect of Glyceraldehyde and Pyruvate on the Activity of the Hexokinase from Rat Skeletal Muscle

Experiment no.	Molar conc. of glyceraldehyde	Molar conc. of pyruvate	Normal γ of glucose disappearing	Per cent inhibition		
				With glyceraldehyde	With pyruvate + glyceraldehyde	With pyruvate
1	5.0×10^{-3}		300	76		
2	4.5×10^{-3}	2.0×10^{-3}	350	100	49	0
	4.5×10^{-3}	4.0×10^{-3}	350	100	50	
3	2.2×10^{-3}	2.0×10^{-3}	550	100	88	
	1.1×10^{-3}	2.0×10^{-3}	550	63	72	
4	2.2×10^{-3}	2.0×10^{-3}	455	87	76	
5	4.5×10^{-3}	2.0×10^{-3}	410	86	92	
6	4.5×10^{-3}	2.0×10^{-3}	240	58	41	0

0.8 ml. of rat skeletal muscle extract was used in these experiments. Other conditions as described in footnote to Table I.

the hexokinase in muscle seems to be the most sensitive of all the enzymes obtained from the various sources mentioned above.

In general, pyruvate does not appear to have any significant effect, although in Expt. 2 (Table VI) it decreased the inhibition from 100% to 49%. It is interesting to note that doubling the concentration of pyruvate had no further effect. It was also found that pyruvate alone had little influence on hexokinase activity, nor could any evidence be uncovered to indicate that there was an increased disappearance of glyceraldehyde in the presence of pyruvate. Further studies showed that the breakdown of hexosediphosphate to lactic acid in the same muscle extracts used above was unaffected by 3×10^{-3} *M* glyceraldehyde.

TABLE VII

The Effect of Increasing Concentrations of Adenosine Triphosphate (ATP) on the Inhibition of Muscle Hexokinase by Glyceraldehyde

Experiment no.	3.0 micromoles ATP		6.0 micromoles ATP		10 micromoles ATP		20 micromoles ATP	
	Normal	Inhibition	Normal	Inhibition	Normal	Inhibition	Normal	Inhibition
		per cent		*per cent*		*per cent*		*per cent*
1					650	83	595	80
2	300	67	420	62	510	66		
3	147	100	320	65	320	82		
4	200	78	255	100	—	—	385	88
5	107	57	265	82				
6	115	100	215	90	—	—	434	77

Conditions similar to those listed under Table VI. Concentration of glyceraldehyde = 1.2×10^{-3} *M*. Total volume in vessels = 2.35 ml.

Other investigators (20,17) have found that increases in the concentration of adenosine triphosphate could decrease the effect of various inhibitors on the hexokinase reaction. This point was tested with muscle extracts and the results are shown in Table VII. From this table it can be seen that an increase in adenosine triphosphate concentration of from 3 to 20 micromoles/2.35 ml. does not relieve an existing inhibition by glyceraldehyde. A decrease of an inhibition of 100% to 77% occurred in only one experiment, while in the others the variations can be accounted for as being within the limits of the experimental error.

The Relationship between the Concentration of Enzyme and the Inhibitory Action of Glyceraldehyde on Hexokinase Activity

Stickland (8) found that the inhibition of glucolysis of muscle extracts fortified with yeast hexokinase could be reversed if the hexokinase concentration was slightly increased. Thus, in one instance an increase in enzyme concentration of 14% reduced an inhibition of 90% to one of 50%. Stickland stressed these experiments as indicating that the concentration of hexokinase was the limiting factor in determining the extent of the action of glyceraldehyde on this enzyme. The same author was also unable to obtain a direct relationship between the enzyme concentration and the measure of enzyme activity as determined by the rate of glucolysis. In the present investigation experiments were performed on the hexokinase reaction in rat skeletal muscle, yeast and tumor extracts, to test these points.

It was observed that, under the conditions adopted in these experi-

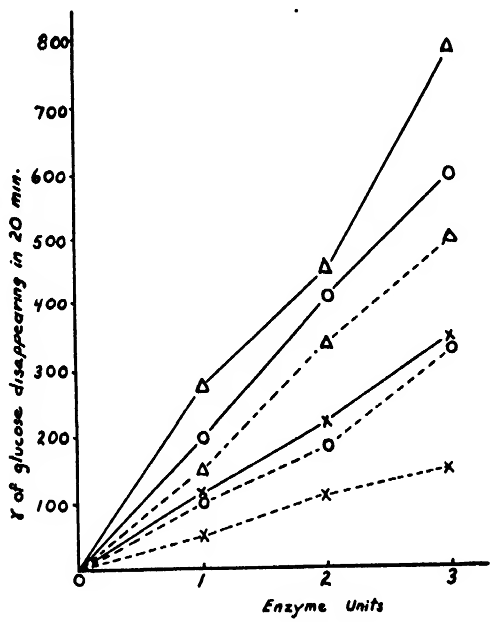

FIG. 1. Solid lines indicate normal, while dotted lines indicate normal + glyceraldehyde. × = Rat skeletal muscle extract. Glyceraldehyde concentration = 1.0×10^{-3} *M*. 1 enzyme unit = 0.8 ml. of extract. O = Aqueous extract of sarcoma 39 acetone powder. Glyceraldehyde concentration = 4.5×10^{-3} *M*. 1 enzyme unit = 0.4 ml. of extract. Δ = Yeast hexokinase extract diluted ten times. Glyceraldehyde concentration = 9.0×10^{-3} *M*. 1 enzyme unit = 0.1 ml. of diluted extract.

ments, a direct linear relationship was obtained between the amount of glucose phosphorylated and the concentration of the hexokinase extract (Fig. 1). Furthermore, an increase in the enzyme concentration did not reverse the inhibition out of all proportion to the amount added. A possible explanation of the differences between the results of the present investigation and those obtained by Stickland might lie in the different experimental conditions adopted.

Discussion

The foregoing experiments demonstrate that *l*-glyceraldehyde inhibits the activity of mammalian tissues. The fact that concentrations of glyceraldehyde smaller than those required to inhibit the hexokinase in extracts of brain and tumor can completely inhibit the glucolysis of these tissues can be explained in several ways.

1. It is possible that other enzymes in the glycolytic cycle beyond the hexokinase stage may be even more sensitive to glyceraldehyde than the hexokinase, *e.g.*, fructokinase (22). On the other hand, it has been shown by various authors (3,21) and verified in this study with muscle extract, that the breakdown of hexose diphosphate to lactic acid is not inhibited by glyceraldehyde. However, such experiments do not offer conclusive proof that all enzymes beyond the aldolase stage in the glycolytic cycle are not affected by glyceraldehyde for the following reasons:

a. It has not been shown in the above-mentioned experiments that the breakdown of hexose diphosphate to lactic acid was operating at a rate comparable to that of glycolysis. Thus, under conditions where the rate of breakdown of hexose diphosphate to lactic acid is less than that observed in glucolysis, there might be a partial inhibition by glyceraldehyde of one of the enzymes involved, yet this inhibition will not be observable in the process hexose diphosphate $\longrightarrow$ lactic acid, because the reaction catalyzed by the inhibited enzyme is not the rate-determining step.[5]

b. It may be that glyceraldehyde acts by uncoupling the oxidative phosphorylation step in glucolysis. If such were the case, the breakdown of hexose diphosphate to lactic acid would remain unaffected, yet there would be observed an inhibition of glucose breakdown, since

[5] The author wishes to thank Dr. M. F. Utter of the Deaprtment of Biochemistry, Western Reserve University, for bringing this point to the author's attention.

the regeneration of adenosine triphosphate would be decreased by the uncoupling, thus leading to a decreased phosphorylation of glucose.[5]

2. The inhibition may be of the type noted by Racker and Krimsky (23), wherein a partial inhibition of an energy-yielding reaction in the glycolytic cycle led to a greatly increased inhibition of glycolysis as measured in terms of lactic acid production.

3. The proteins of the extract may exert a protective effect on the hexokinase.

The mechanism whereby pyruvate reverses the inhibition of glycolysis by glyceraldehyde still remains obscure. The foregoing experiments show that pyruvate does not act directly on the hexokinase in tissue extracts. It is possible that the enzymes mediating the pyruvate effect are destroyed in tissue extracts, or that pyruvic acid can bring about the removal of *l*-glyceraldehyde in quantities sufficient to lift the inhibition. The method at our disposal could not decide this point. Increased amounts of pyruvate appearing under aerobic conditions in tissue slices might also explain why concentrations of glyceraldehyde which inhibit glycolysis do not inhibit respiration.

Acknowledgment

The author wishes to express his thanks to Dr. Bruno Mendel for his advice and encouragement during the course of this work.

Summary

To determine the mechanism whereby glucolysis is inhibited by *dl*-glyceraldehyde, the effect of this substance was tested on the activity of the enzyme hexokinase obtained from various sources, such as rat skeletal muscle, rat sarcoma 39, beef brain and yeast, with the following results:

1. The hexokinase from the above-mentioned sources is inhibited by *dl*-glyceraldehyde and the inhibition is caused by the *l*-isomer.

2. The sensitivity of the enzyme to the inhibitor varies with the source of the enzyme; thus, muscle hexokinase activity is inhibited completely by 2×10^{-3} M *dl*-glyceraldehyde, while that from yeast is only partially inhibited by 10^{-2} M.

3. Aqueous extracts of acetone powders of rat sarcoma 39 are more sensitive to the inhibitor than are homogenates.

4. Increasing the concentration, either of the hexokinase or of adenosine triphosphate, does not affect the extent of the inhibition.

5. Under the experimental conditions adopted, pyruvate does not reverse the inhibitory effect of glyceraldehyde on hexokinase activity from various sources. On the other hand, pyruvate does abolish the inhibition of anaerobic glucolysis by glyceraldehyde in slices of rat sarcoma 39.

6. The amounts of glyceraldehyde required to inhibit glucolysis in slices are smaller than those required to inhibit the hexokinase reaction in extracts.

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Distribution of Thiamine in the Brain

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Introduction

Peters and associates have shown that cocarboxylase (thiamine pyrophosphate) is an essential factor in pyruvate oxidation in the brain calling attention to the importance of vitamin B_1 in nerve function (1). More recently, the findings of Minz and of von Muralt have revealed a possible new role played by thiamine in the mechanism of nerve impulse transmission (2,3). Vianna Dias has shown that thiamine, when administered locally to the brain, produces a very marked excitatory effect (4). This property is specific for the entire thiamine molecule and gives evidence of its possible action on the central nervous system. Moreover, it is known that thiamine inhibits cholinesterase and reinforces many of the effects of acetylcholine (5,6).

As acetylcholine and cholinesterase are distributed differently in the various parts of the brain (7,8), it would be desirable to know whether thiamine has a similar distribution. The published values refer only to the thiamine content of the whole brain; analytic data for the thiamine content of the white and gray matter are still lacking. Leong, determining the thiamine of rat brain by the bradycardia method found 2.0 γ/g. of fresh tissue (9). Ochoa and Peters give values for the rat and the pigeon which varied from 2.7 to 3.6 γ, expressed as cocarboxylase (10). Williams and coworkers reported 4.1–4.8 γ for the rat, and 1.4–1.8 γ/g. for the human brain (11). Muralt, working with the thiocrome and *Phycomyces* tests obtained a value of 2.6 γ for the gray matter (12).

The present paper deals with the results obtained for the white and gray matter (cortex) and for the nucleus caudatus of normal dogs. Some experimental data on animals injected with large doses of thiamine are also included. The thiamine of the nervous tissues of dogs under varied conditions is being studied and will be reported upon later.

Material and Methods

Twenty-three adults dogs weighing 8–16 kg. were used. The animals were killed by intravenous injection of chloroform (about 2. ml) and the brain removed as soon as possible and cleaned from contaminating blood. We observed that chloroform causes the brain vessels to empty and therefore avoids undesirable accumulation of blood. The hemispheres were cut in thin slices and the gray matter was separated from the white mass using a sharp knife and a razor blade. Nucleus caudatus was also removed and weighed separately. For the analysis, 250 mg. to 1 g. of tissue was weighed on an analytical balance and ground in an agate mortar until a fine homogeneous mass was obtained. Small volumes (1 ml. each) of 0.1 N H_2SO_4 were added and the mass well mixed. The resulting suspension was carefully transferred to a Pyrex test tube and made up to 5 ml. with acid solution. The tube was left in a boiling water bath for 15 min. After cooling, 2 ml. of a 10% suspension of takadiastase (Parke, Davis & Co.) was added and the solution adjusted to pH 4.5. The solution was then incubated 24 hr. at 37°C. After hydrolysis, the solution was filtered and clarified with Celite (Hyflo-supercel) and the volume made up to 10 ml. with distilled water. The clear filtrate was used for the thiamine determinations.

Sarett and Cheldelin's microbiological method using *Lactobacillus fermentum 36* was employed for all determinations (13). In some cases, the slant cultures were fortified with high doses of thiamine as recommended by Cheldelin *et al.* (14). Each assay was duplicated and accompanied by an assay with a standard thiamine solution. The thiochrome method of Hennessy and Cerecedo, slightly modified, was performed on only 8 samples and the results were in good agreement with the microbiological determinations. The fluorescence of the thiochrome was measured in a Pfalz and Bauer fluorometer and the adsorption run in a Decalso column (15).

Thiamine hydrochloride[1] dissolved in 0.85% saline was injected subcutaneously daily into 6 normal dogs and the animals killed with chloroform. The animals were sacrificed one day after the last injection. Four dogs were injected daily with 1 mg. of thiamine/kg. body weight, while 2 others received, respectively, 8.5 and 10.0 mg./day.

Results

In Table I are shown the values obtained in 17 normal dogs for the thiamine content of the gray matter (cortex), white matter and nucleus caudatus. The average values for the white matter revealed lower amounts of this vitamin than for the cortex and nucleus caudatus. The same distribution was maintained in the brain of the dogs saturated with thiamine (Table II). Probably the variations encountered in the normal dogs are probably due in part to the varied origin of these animals, since they were of different breeds and environmental conditions which may have affected their nutritional state.

[1] Thiamine hydrochloride was kindly supplied by Hoffman La Roche, Rio de Janeiro, to whom we are grateful.

The storage of thiamine in the brain seems not greatly affected when large doses of thiamine are administered. Leong reported that the rat fed a diet rich in vitamin B_1 attained a maximum storage with about 30 I. U. of thiamine per day (9). Ochoa and Peters similarly observed that the thiamine content of the total brain reaches a level not far beyond the normal in animals receiving extra doses of this vitamin (10). In our experiments, where large amounts of thiamine were administered, the values increased to only slightly over the normal average, suggesting that the brain is nearly saturated with thiamine. Thus, dogs 4 and 5, which received 8.5 and 10.0 mg. of thiamine per day, presented a thiamine content comparable with those injected with lower doses and only slightly higher than some of the normal dogs.

TABLE I

Thiamine Distribution in the Brain of Normal Dogs

Total thiamine in γ/g. of tissue

No. of dogs	Gray matter	White matter	Nucleus caudatus
1	2.8	1.1	2.7
2	1.7	0.7	2.1
3	1.9	1.4	2.3
4	2.4	1.6	3.4
5	1.3	0.8	1.3
6	2.7	1.2	2.3
7	1.7	1.3	1.7
8	1.3	1.0	2.2
9	1.4	2.0	1.5
10	1.4	1.3	1.6
11	2.1	0.8	2.4
12	2.1	1.3	1.5
13	1.6	0.7	1.4
14	2.0	1.5	1.7
15	1.3	1.0	2.1
16	1.3	1.1	1.9
17	1.7	1.2	1.8
Mean and S.D: (σ)	1.8±0.48	1.2±0.37	2.0±0.53
Stand. error:	0.12	0.09	0.13

$$\sigma = \sqrt{\frac{\Sigma\,(x)^2}{n-1}} \quad \text{Stan. error} = \frac{\sigma}{\sqrt{n}}.$$

TABLE II

Thiamine Content of the Brain of Injected Dogs

Dogs	Thiamine inj. mg./kg. body wt./day	Time in days	Thiamine in γ/g. of tissue		
			Cortex	White matter	Nucleus caudatus
1	1	21	2.6	1.7	2.9
2	1	10	3.2	1.2	4.0
3	1	13	3.0	3.0	3.1
4	1	13	2.7	2.1	4.0
5	8.5	3	2.7	2.5	3.6
6	10.0	8	2.3	2.1	2.6
Mean and S. D. (σ)			2.7±0.32	2.1±0.62	3.4±0.62
Stan. error			0.14	0.28	0.28

Summary

Thiamine was determined in the brain of 17 normal dogs and showed a varied distribution. The values averaged 1.8 $\pm$ 0.48 γ for the cortex, 1.2 $\pm$ 0.37 γ for the white matter, and 2.0 $\pm$ 0.53 γ/g. of tissue for the nucleus caudatus. The injection of large doses of thiamine hydrochloride subcutaneously provoked a limited increase of this vitamin in the brain with a distribution similar to that presented by the normal uninjected animals.

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The Sulfur Amino Acid Requirement of *Tetrahymena geleii* [1]

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Introduction

Recently, a paper by Kidder and Dewey (1) showed that, on a methionine-low medium, *Tetrahymena geleii* W, a ciliated protozoon, gave a growth response to the addition of homocystine to the medium in the presence of liver extract.[2] They explained their results on the basis of the methylation of homocysteine to form methionine through the mediation of a factor in the liver which they believed could be described as a co-transmethylase. They also reported that homocystine plus choline did not permit growth of *Tetrahymena* in the absence of the liver factor. Furthermore, they stated that exogenous choline did not influence the methylation of homocystine. To account for a source of methyl groups, they assumed the synthesis of the methyl group from some unknown precursor. The small amount of methionine in their liver extract was reported to be insufficient to explain their results.

In view of the fact that dimethylthetin was a methyl donor for the rat, it occurred to us that perhaps dimethylthetin might possibly serve as a methyl donor for *Tetrahymena*. Experiments were undertaken to test this possibility. Dimethylthetin, however, was unable to act as a methyl donor when added to increasing amounts of homocystine in the presence of the liver extract. Furthermore, choline, betaine and dimethylpropiothetin were equally ineffective although the quantities added were sufficient to combine with all of the homocystine present.

We then turned to the examination of the liver factor. Kidder and Dewey found that, in the presence of the liver extract (L.E.L.) and graded amounts of homocystine, the organism showed a growth effect greater than could be accounted for on the basis of the methionine content of the medium and the added liver extract. Since they could not account for this additional growth on the basis of the methionine present, they

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[1] The author wishes to thank the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

[2] Liver Extract Lilly No. 343, referred to as L.E.L.

assumed that additional methionine must have been formed from the homocystine present.

When we attempted to reproduce this growth effect, we obtained only as much growth as could be accounted for on the basis of the methionine present in the particular samples of Liver Extract Lilly No. 343 we had obtained. However, our method of assay for methionine was different, as will be explained later. We suspected that one might be dealing with a simple question of requirement of sulfur amino acids in the presence of a minimal required amount of methionine. Homocystine might thus be supplying the remaining requirement for organic sulfur. If this were true, cystine might be capable of replacing homocystine in the presence of this minimal amount of methionine. And indeed, this proved to be the case, for cystine plus liver extract, or an amount of methionine equivalent to that present in the liver extract, gave just as good growth as was obtained with homocystine plus liver extract.

Obviously, one was not dealing with a methylation problem involving a co-transmethylase, but rather with the significance of total amounts of sulfur amino acids to the growth of *Tetrahymena*. The situation would seem to be quite comparable to that in the rat (2) and in the chick (3), where cystine gives an increased growth effect when added to a limited amount of methionine in the diet.

The most probable explanation for the differences between our results and theirs lies in the methionine assay data. According to Kidder and Dewey, the amount of L.E.L. they added contained 0.4 γ of L-methionine as assayed by a method using *Leuconostoc mesenteroides*, whereas our value for the same weight of L.E.L. was 1.4 γ of L-methionine. In our assay method *Streptococcus faecalis* was used as the test organism but similar values were also obtained when *Tetrahymena* itself was used as the assay organism. Furthermore, when a much earlier preparation of L.E.L. was tested, methionine values of the same order of magnitude were obtained. If one assumes that the methionine value for the liver extract used in the experiment by Kidder and Dewey was the same as we found, the excess growth they obtained could be explained almost completely on the basis of the methionine content of the liver extract and medium.

If our explanation of the phenomenon be correct, the elaborate theories of transmethylation proposed by Kidder and Dewey for *Tetrahymena* and for higher forms of animal life derive no support from their experimental results on *Tetrahymena*.[3]

[3] Prior to submission to the journal, this paper was sent to Dr. Kidder for his criticism. He has informed us that they have re-evaluated the methionine content of their liver fraction using *Tetrahymena* as the assay organism. He stated that, using their purified medium and supplying the Factor II by the addition of protogen [Stokstad

Materials and Methods

Organism and Inoculum

Tetrahymena geleii W,[4] the ciliated protozoon used for these experiments, was maintained on a liquid medium composed of 1% dextrose, 1% peptone (Bacto), and 1% yeast extract. The stock culture was transferred at monthly intervals and was incubated and stored at 25°C.

The cultures for inoculum were grown for 2 or 3 days in an unslanted position. For inoculation of the experimental tubes 1 drop of a 1–10 dilution in saline was added to each tube and the tubes were incubated at 25°C. for 6 days in a *slanted* position according to the method of Kidder and Dewey. Readings were made with a Klett-Summerson colorimeter using a No. 66 filter (640–700 mμ).

Medium

The medium was prepared according to the formula of Kidder and Dewey (1) and the pH was adjusted to 7.0 before the medium was autoclaved. It consisted of amino acids, with the exception of the sulfur-containing amino acids, 1% glucose, vitamins including 1 γ/ml. of choline, hydrolyzed yeast nucleic acid and Cerophyl[5] at a 1–5 dilution. In some experiments, the choline was omitted and the Cerophyl was used at a 1–10 dilution. The hydrolyzed yeast nucleic acid was prepared as follows: 10 g. of yeast nucleic acid were dissolved in 50 ml. of water and 5 ml. of 25% NH_4OH and autoclaved in a sealed tube for 1 hr. at 118°C. according to the method of Levene (5); after removal of the ammonia under reduced pressure, the pH of the solution was adjusted to 7.0 and the solution was filtered and diluted to 500 ml. Whenever necessary, the medium or its ingredients were stored with chloroform and toluene.

The medium, prepared in double strength lots, and addenda were placed in a tube 20 × 150 mm. to make a total volume of 6 ml. The tubes were plugged with cotton and autoclaved for 6 min. at 15 lbs. pressure.

Three samples of liver extract were used; two were from Lilly and Company[6] and one was from Lederle Laboratories[7]. The Lilly samples, Liver Extract No. 343, were

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et al., Arch. Biochem., **20,** 75 (1949)], their assay results for methionine in the liver fraction which they used turned out to be 15 γ/mg. or 1.8 γ/ml. on the 120 γ of L.E.L. used. Recovery experiments averaged 94%. When cysteine was added to the assay medium, they obtained 10.8 γ/mg., or 1.3 γ/ml. on the 120 γ of L.E.L. used. A recovery value of 106% was obtained in the latter case. Dr. Kidder has authorized us to state that they are in full accord with the results expressed in this paper and to include the above data in a footnote to this paper.

[4] This organism was obtained through the kindness of Dr. E. L. R. Stokstad of Lederle laboratories, Pearl River, N. Y.

[5] The Cerophyl was obtained through the courtesy of Cerophyl Laboratories, Inc., Kansas City, Mo.

[6] The recent Lilly sample was obtained through the courtesy of Dr. E. D. Campbell, Eli Lilly and Co., Indianapolis, Ind.

[7] The Lederle Liver Extract was furnished by Lederle Laboratories, Pearl River, N. Y.

of two Lot Numbers, 209S—861400 and 436627. The Lederle preparation was Solution Liver Extract—3.3 U. S. P. injectable units per ml. prepared for intramuscular injection. The pH of the Lilly samples was adjusted to 7.0 before they were added to the medium. All liver extracts were added in a volume of 0.1 ml. to 6 ml. of medium.

L-Methionine Assay

A microbiological method was used for the determination of the L-methionine content of the liver samples and Cerophyl preparations. The earlier assays were performed by the method of Stokes *et al.* (5) and the later ones by the method of Henderson and Snell (6). The same organism was employed for both assays, namely, *Streptococcus faecalis*, ATCC No. 8043. The Lilly extracts were assayed before and after an 18 hr. hydrolysis in 6 *N* HCl, but the Lederle Liver Extract and the Cerophyl were assayed as used.

Experimental Results

The first evidence to be presented is an experiment designed to duplicate the findings obtained by Kidder and Dewey. The results are summarized by the curves in Fig. 1. The constant amount of homocystine used in the preparation of Control Curve 3 was the amount chosen by Kidder and Dewey as giving maximal stimulation under the conditions of their experiment.

All values plotted in Fig. 1 were obtained after 3 serial transfers through the same medium. This technique involved an original inoculum as described above plus two more transfers through the same concentration of test substances, *e.g.*, control tube to control tube, 1 γ of methionine to 1 γ of methionine, *etc.* Readings were made after each 6 day growth period. The value of the serial transfer technique is questionable for this experiment. The only difference obtained after serial transfer was a drop of 10–15 colorimeter units in the readings of tubes containing small amounts of methionine, as was the case with the values of Control Curve 1. However, the technique was applied in order to parallel the work of Kidder and Dewey as closely as possible.

The growth response to increasing amounts of homocystine in the presence of constant amounts of the liver extract, Curves A and B, is paralleled by that of Control Curve 2 where the constant amount of liver extract is replaced by an amount of methionine equal to the amount present in 120 γ of hydrolyzed L.E.L. Lot No. 436627. On the basis of methionine assay values of the hydrolyzed material, 120 γ of L.E.L. Lot No. 436627 added 1.4 γ of methionine to the medium and 120 γ of Lot No. 209S added 1.2 γ of methionine. Control Curve 2 shows that the L.E.L. is causing no greater amount of growth than that which would be expected on the basis of its methionine content.

There is one point on Control Curve 3 that is of particular interest to the experiment. The growth represented by this point (marked by a dotted line) is to be compared with that represented by the 40 γ points

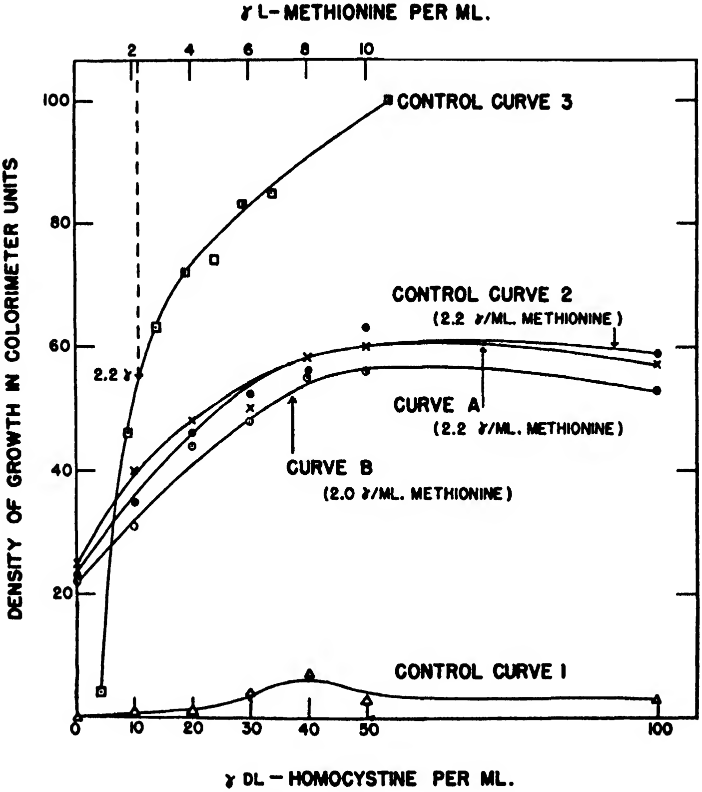

FIG. 1. Utilization of DL-homocystine by *Tetrahymena* in the presence of Liver Extract Lilly (L.E.L.) or L-methionine.

These values were obtained after 3 serial transfers. Control Curve 1: graded amounts of homocystine; Control Curve 2: graded amounts of homocystine + 2.2 γ of methionine (0.8 γ from the Cerophyl of the medium and 1.4 γ of added methionine); Curve A: graded amounts of homocystine + 120 γ/ml. of L.E.L. No. 343, Lot 436627 (methionine content = 2.2 γ: 0.8 γ from the Cerophyl + 1.4 γ from L.E L.); Curve B: graded amounts of homocystine + 120 γ/ml. of L.E.L. No. 343, Lot 209S-861400 (methionine content = 2.0γ : 0.8 γ from the Cerophyl + 1.2 γ from L.E.L.); Control Curve 3: graded amounts of methionine + 40 γ/ml. of homocystine. The growth values are plotted at the level of methionine represented by the amount of methionine added per ml. + 0.8 γ of methionine from the Cerophyl. The point on Control Curve 3 shown by the dotted line indicates growth at a level of 2.2 γ of methionine and 40 γ of homocystine. This amount of growth corresponds to the amount of growth on Control Curve 2 and Curve A at the point of 40 γ of homocystine.

on the homocystine curves—Curve A and Control Curve 2—because all 3 of these points represent the growth resulting from the addition of 2.2 γ of methionine and 40 γ of homocystine to the medium. Since

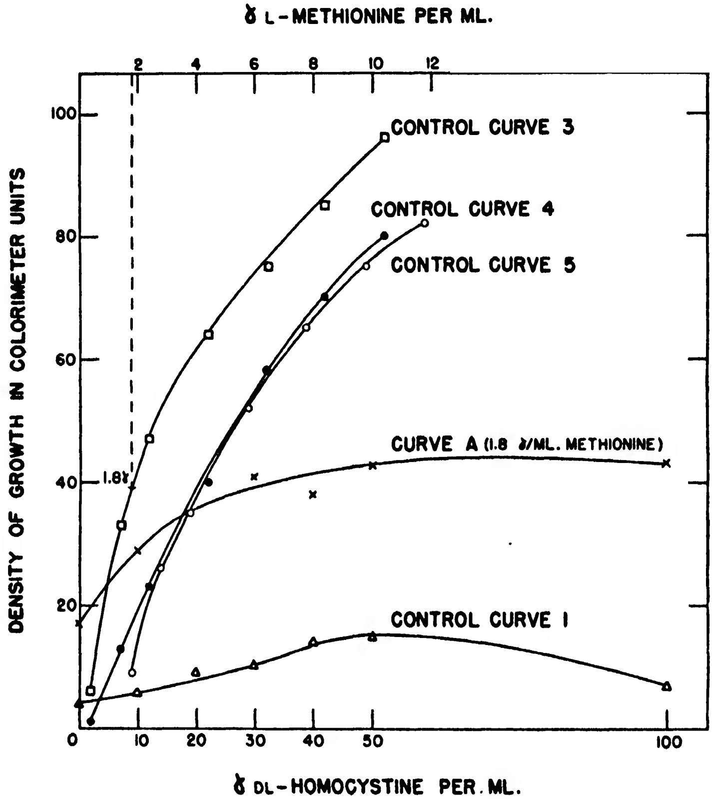

FIG. 2. Utilization of DL-homocystine by *Tetrahymena* in the presence of Lederle Liver Extract.

The values were obtained without serial transfer. Curve A: graded amounts of homocystine + 0.0007 ml. of Lederle Liver Extract (methionine content = 1.8 γ: 0.4 γ from the Cerophyl + 1.4 γ from the Lederle Liver Extract); Control Curve 1: graded amounts of homocystine; Control Curve 3: graded amounts of methionine + 40 γ/ml. of homocystine. The point on Control Curve 3 shown by the dotted line indicates growth at a level of 1.8 γ of methionine and 40 γ of homocystine. This amount of growth corresponds to the amount of growth on Curve A at the point of 40 γ of homocystine. Control Curve 4: graded amounts of methionine; Control Curve 5: graded amounts of methionine + 0.0007 ml. of Lederle Liver Extract. In Control Curves 3, 4, and 5, the growth values are plotted at the level of methionine represented by the amount of methionine added per ml. + 0.4 γ of methionine from the Cerophyl. In Control Curve 5, an additional 1.4 γ of methionine has been added, due to the amount present in the liver extract.

these points on Curves A and B are about opposite the corresponding points on Control Curve 3, there is obviously no extra growth over and above that which can be attributed to the methionine and homocystine present. This is in sharp contrast to the results reported by Kidder and Dewey. They obtained a great deal more growth in the presence of 40 γ of homocystine plus 120 γ of L.E.L. than that observed in the presence of the same amount of homocystine plus an amount of methionine corresponding to the amount in 120 γ of L.E.L.

The previous experiment has shown that two different samples of L.E.L. gave a utilization of homocystine that could be accounted for by the amount of methionine present in the liver. At an early stage in this work, before L.E.L. was available, a sample of Lederle Liver Extract solution was tried. An amount of Lederle Liver Extract equivalent to 120 γ of L.E.L. was calculated to be 0.0007 ml., on the basis of data given in the paper of Bennett and Toennies (7). In this case the medium was different in two respects from the previous experiment. The Cerophyl was used at a dilution of 1–10 and the choline was omitted. Still another difference from the conditions of the first experiment was that the readings for these tubes were made after 6 days of growth without carrying the experiment through serial transfers.

The results plotted in Fig. 2 with a different liver product, that of Lederle Laboratories, are essentially the same as those found in the serial transfer experiment with L.E.L. When the height of Curve A at 40 γ of homocystine is compared to the point on Control Curve 3 at 1.8 γ of L-methionine, the levels are found to be about the same. There is no extra growth over and above that which can be accounted for by the amount of L-methionine in the Lederle Liver Extract. The fact that Control Curves 4 and 5 are practically coincidental when plotted shows that the liver is adding no unknown growth factor.

Although these results were obtained with a different liver extract, after a single inoculation, without serial transfer, and in the absence of added choline, they are similar to those obtained with L.E.L.

Since we had found that methionine could substitute for the liver extract in the presence of increasing amounts of homocystine, it seemed of interest to attempt to substitute cystine for the homocystine. The data for this experiment have been summarized in the 4 curves of Fig. 3 which are similar to those for the homocystine experiment (Fig. 1). Only one liver extract, L.E.L. Lot No. 436627, was used. A slightly greater amount of L-methionine, 1.5 γ/ml., replaced the L.E.L. in obtaining the values plotted in Control Curve 2, although 1.4 γ/ml. of L-methionine is probably the value closer to the equivalent amount of

methionine. The medium was the same as that for the homocystine experiment and this experiment was likewise carried through 3 serial transfers.

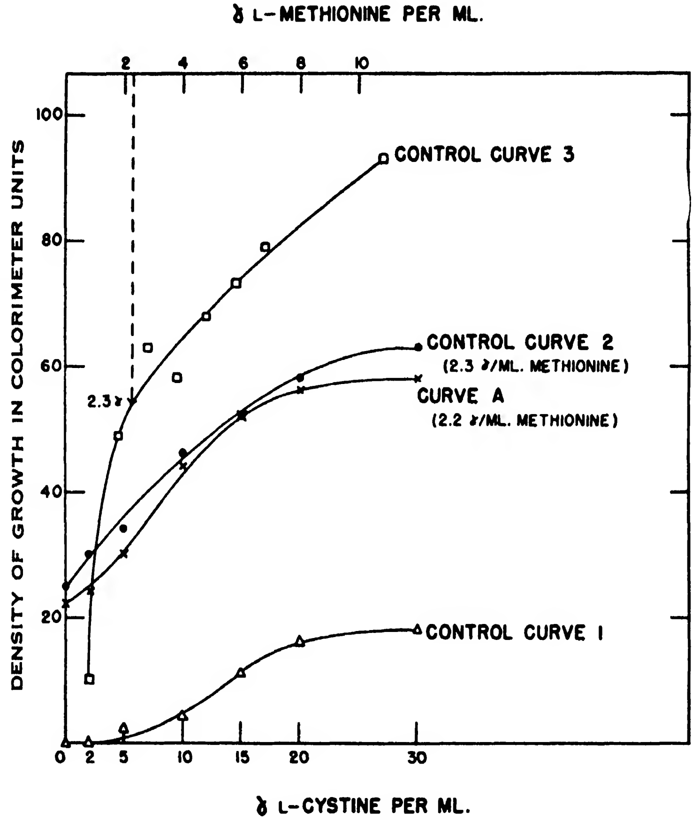

Fig. 3. Utilization of L-cystine by *Tetrahymena* in the presence of Liver Extract Lilly (L.E.L.) or L-methionine.

The values were obtained after 3 serial transfers. Control Curve 1: graded amounts of homocystine; Control Curve 2: graded amounts of homocystine + 2.3 γ of methionine (0.8 γ from the Cerophyl of the medium and 1.5 γ of added methionine); Curve A: graded amounts of homocystine + 120 γ/ml. of L.E.L. No. 343, Lot 436627 (methionine content = 2.2 γ: 0.8 γ from the Cerophyl + 1.4 γ from L.E.L.); Control Curve 3: graded amounts of homocystine + 20 γ/ml. of L-cystine. The growth values are plotted at the level of methionine represented by the amount of methionine added per ml. + 0.8 γ of methionine from the Cerophyl. The point on Control Curve 3 shown by the dotted line indicates growth at a level of 2.3 γ of methionine and 40 γ of homocystine. This amount of growth may be compared to the amount of growth on Control Curve 2 and Curve A at the point of 40 γ of homocystine.

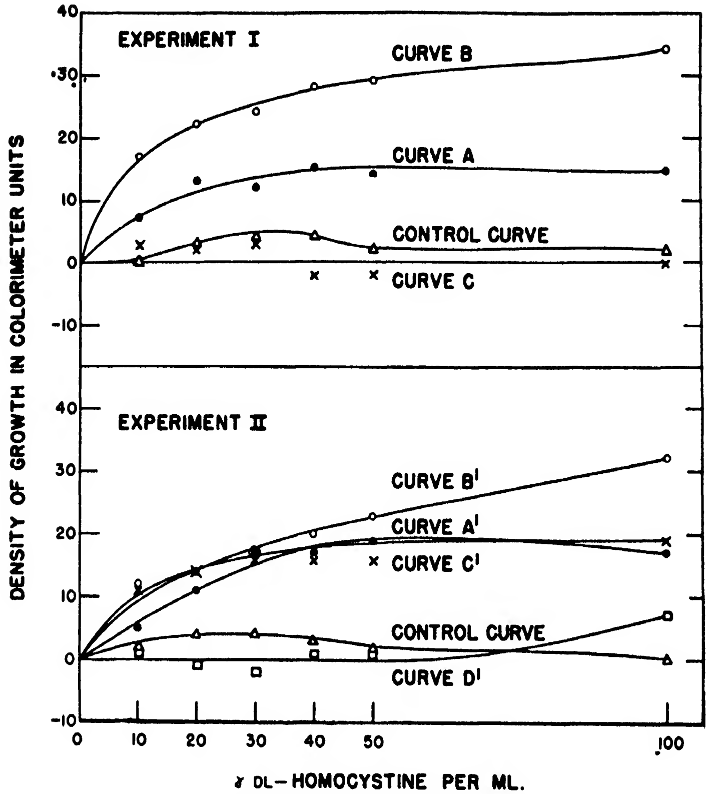

FIG. 4. Effect of increasing levels of Liver Extract Lilly (L.E.L.) or L-methionine upon the utilization of DL-homocystine by *Tetrahymena*.

All values have been plotted after subtraction of the control tube reading. *Expt. I:* Control Curve: graded amounts of homocystine; Curve A: graded amounts of homocystine + 100 γ/ml. of L.E.L. No. 343, Lot 436627 (methionine content = 1.2 γ); Curve B: graded amounts of homocystine + 400 γ/ml. of L.E.L. (methionine content = 4.8 γ); Curve C: graded amounts of homocystine + 1000 γ/ml. of L.E.L. (methionine content = 12 γ). *Expt. II:* Control Curve: graded amounts of homocystine; Curve A′: graded amounts of homocystine + 1 γ/ml. of methionine; Curve B′: graded amounts of homocystine + 4 γ/ml. of methionine; Curve C′: graded amounts of homocystine + 10 γ/ml. of methionine; Curve D′: graded amounts of homocystine + 20 γ/ml. of methionine.

The results show that L-cystine can replace DL-homocystine in the presence of either L.E.L. or an equivalent amount of methionine. The Control Curves 2 and 3 of Fig. 3 bear the same relationship to Curve A as did the Control Curves 2 and 3 of Fig. 1 to Curves A and B.

Further evidence that the liver extract or an equivalent amount of

methionine stimulate the utilization of graded amounts of homocystine in a similar manner is presented in Fig. 4. The amounts of methionine or liver extract in the medium were maintained at given levels and the homocystine was added in graded amounts. The final medium contained Cerophyl at a dilution of 1–10 but no added choline. In plotting the curves of Fig. 4, the values from the control tubes were used as the zero point in order to compare more readily the results at the various levels of liver extract (Fig. 4, Expt. I) or methionine (Fig. 4, Expt. II).

There is a relative increase in the utilization of the homocystine with amounts of methionine up to 4 γ/ml. and with amounts of liver up to 400 γ/ml. equivalent to 4.8 γ/ml. of methionine. At 10 γ/ml. of methionine the organism can no longer use the homocystine as efficiently as it uses it at 4 γ/ml., and at the 20 γ/ml. level of methionine there is no increase in growth, with amounts of homocystine up to 50 γ/ml., over that in the control tube containing methionine alone. However, the level of 1000 γ/ml. of liver, which contains only 12 γ/ml. of methionine, gives a curve very similar to that given by 20 γ/ml. of methionine. This is due in part, we believe, to the cystine content of the L.E.L. One thousand γ of L.E.L. would contain 5 γ of cystine, on the basis of an L.E.L. analysis (7). This cystine would add to the stimulating effect of the methionine present and so cause Curve C (Fig. 4, Expt. I) to approach the curve of the 20 γ/ml. level of methionine (Curve D′, Fig. 4, Expt. II).

The results of this experiment show that with 1 γ/ml. of methionine in the medium there is a limited use of increasing amounts of homocystine. With about 4 γ/ml. of methionine, there is a maximal use of homocystine, and with increasing amounts of methionine, up to 20 γ/ml., there is a diminishing use of homocystine.

Similar results were obtained when the growth on increasing amounts of methionine, up to 30 γ/ml., was compared with the growth on increasing amounts of methionine plus 40 γ/ml. of homocystine. At a level of 15–20 γ/ml. of methionine, the amount of growth was the same, with or without added homocystine.

When a limited amount of methionine is present, increased growth of *Tetrahymena* is observed in the presence of added homocystine. However, with a maximal amount of methionine present, addition of homocystine does not result in any additional growth of *Tetrahymena*. Comparable results are obtained when cystine replaces homocystine.

Acknowledgment

The author wishes to express appreciation for the invaluable advice and counse given by Dr. Vincent du Vigneaud throughout the course of this work. The capable

assistance of Miss Mary R. Lloyd and Mrs. Susan M. Wing in the microbiological work, and of Mrs. Josephine T. Marshall in the preparation of the figures is gratefully acknowledged.

Summary

We have been unable to confirm the findings of Kidder and Dewey upon which they base their contention that a co-transmethylase is partly responsible for the utilization of homocystine by *Tetrahymena geleii* W in the presence of Liver Extract Lilly No. 343. In our experiments, the increased growth of *Tetrahymena* in the presence of graded amounts of homocystine and a constant amount of liver extract seemed to be due entirely to the methionine content of the liver extract and medium. Furthermore, it was found that cystine could replace homocystine in the nutrition of *Tetrahymena* under similar conditions.

The response of *Tetrahymena* to increasing amounts of homocystine or cystine in the presence of limited amounts of methionine appears to depend upon an overall deficiency of sulfur amino acids. In the presence of an amount of methionine up to 15 γ/ml., addition of cystine or homocystine resulted in an increased amount of growth. In the presence of maximal amounts of methionine, addition of cystine or homocystine did not increase the growth of *Tetrahymena*.

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Concentration of Amino Acids by the Excised Diaphragm Suspended in Artificial Media. I. Maintenance and Inhibition of the Concentrating Activity [1]

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Introduction

This communication reports a study *in vitro* of the activity of cells in maintaining internal concentrations of amino acids much higher than those found in the fluids which bathe them (1,2). The ability of the excised diaphragm of the rat and of the guinea pig to concentrate glycine has been maintained during 1–3 hours of immersion in saline-bicarbonate solutions containing sodium pyruvate. The influences of various factors, and of various substances added to these media, upon the assimilative activity have been studied. Some of the conclusions reached were confirmed when the distribution of total α-amino acid nitrogen instead of glycine was studied.

The concentrative assimilation of glutamic acid by brain cortex slices has been described by Stern (3) in a preliminary communication. The disappearance of glutamic acid from the suspending fluid was accounted for partially by the accumulation of glutamic acid within the slices and partially by chemical change of the glutamic acid. The assimilation occurred in the presence of glucose or certain other substrates, and was inhibited by malonate, azide, luminal, and iodoacetate, and by the absence of oxygen.

Changes in the amount of free amino acids in the system were small when a hemidiaphragm carefully excised from a rat was shaken in saline solutions under the conditions described here. The diaphragm of the rat

[1] Assisted by a grant-in-aid from the Abbott Laboratories, Inc., North Chicago, Ill.

contained about 5 times as high a glycine concentration as the plasma, on a fresh tissue basis, corresponding to a ratio of about 7.5 for the concentration in the cellular water to that in the extra-cellular water. No assumption is made that the apparently free amino acids of the tissues are dispersed in the cell water.

Experimental

Sprague-Dawley rats weighing 180–210 g. were fasted 20 hr. then anesthetized lightly with ether, and blood was drawn from the heart into tubes containing heparin. The hemidiaphragms were removed quickly with minimal trauma, weighed on a precision torsion balance, and placed at once in 2–2.2 ml. of the freshly prepared medium in a 25 ml. Erlenmeyer flask at room temperature. This was set to shaking in a water bath at 37°C. In some cases, the second half-diaphragm was extracted at once for analysis after weighing (no equilibration in artificial media). The agitation was by horizontal to and fro motion 6.8 cm. long, at 60–78 cycles/min., with a gas mixture containing 95% O_2–5% CO_2 passing through the flask. At the end of the experimental period the hemidiaphragm was blotted on filter paper and extracted carefully with saturated aqueous picric acid (2.5 ml. for every 100 mg. of tissue) by grinding in a mortar. Three aliquots of 1 ml. of filtrate could be obtained, permitting duplicate glycine determinations as well as a blank analysis. When α-amino nitrogen was to be determined, the tissue was extracted repeatedly by grinding with aqueous picric acid, filtering into the special tube for this determination (4), to a total volume of about 5 ml. This filtrate was buffered at pH 6.5 with phosphate and heated 90 min. to eliminate the α-amino group of glutamine and half the α-amino nitrogen of glutathione (5). Plasma and suspending media were deproteinized by adding 5 volumes of picric acid. Whenever α-amino nitrogen was to be determined, these filtrates also were heated 90 min. at 100°C. at pH 6.5.

Glycine was determined by the method of Alexander, Landwehr and Seligman (6), using, however, twice as much ninhydrin as originally recommended. This analytical procedure is discussed in another report (7). The small specimens available necessitated a halving of the volumes used in the glycine analyses of diaphragms. The total volume of distillate collected in the procedure (6) was adjusted to 5 ml. rather than 10 ml. The volume of water added midway in the distillation was usually 1.9 ml. or more. These distillations were completed in about 9 min. Entirely satisfactory results were obtained with this modification. A rough neutralization of the residual picric acid in the filtrates was necessary (about 1 drop 0.75 *N* NaOH/ml. of filtrates of diaphragm and saline media; 1 drop/2 ml. of plasma filtrates and 1:11 tissue filtrates).

Results

When the hemidiaphragm was shaken in a glycine-free salt solution, from half to two-thirds of the diaphragm glycine diffused into the medium in 1 hr. Only when enough glycine was placed in the medium to prevent or diminish this glycine transfer were we able to compare

the activity of various substances in promoting the retention or loss of glycine by the tissue. A fluid glycine concentration approximately equal to that of the plasma prevented the fall of the glycine level of the diaphragm during 1–3 hr. of equilibration in an appropriate medium (see below). If the fluid glycine level was higher than the plasma concentration, glycine was transferred from the fluid into the diaphragm; if lower, glycine was transferred from the diaphragm to the fluid (Table I).

TABLE I

An Experiment Illustrating the Responsiveness of the Diaphragm Concentration to the Level in the Suspending Fluid

Two paired portions of diaphragm were shaken for 2 hr. in media alike except in glycine concentration. A third control portion was analyzed at once upon excision.

	Fluid glycine N	Diaphragm glycine N	Distribution ratio
	mg.-%	*mg.-%*	
In the intact rat	0.48[a]	2.40	5.0
Equilibrated portion 1, after 2 hr.	0.60	2.91	4.9
Equilibrated portion 2, after 2 hr.	0.38	1.95	5.1

[a] Plasma concentration × 1.05.

When the glycine of the medium was made twice as high as that of the plasma, the glycine concentration of the tissue was nearly doubled in 2 hr. of equilibration (Table IV). These results gave some justification for comparing the distribution of glycine between the diaphragm and the plasma on one hand, and between the diaphragm and an artificial suspending fluid on the other. These comparisons have been made in terms of the *distribution ratio*, that is, the ratio of the diaphragm concentration (fresh weight basis except as indicated) to extracellular concentration.

The total amount of "free" glycine nitrogen in the system, hemidiaphragm-suspending fluid, was increased on an average by only 0.8 γ or 6% during 1 or 2 hr. of incubation in glycine-containing media (27 Expts.). The small amount of extra glycine may have been formed by either cellular or extracellular proteolysis; experiments with tissue slices have suggested that the cut boundaries may have been a factor.

One could be surer of how strongly a substance affected glycine assimilation using a second technique. Two paired diaphragms were shaken simultaneously in two sets of media, alike except in one ingredient. The final glycine concentrations of the two

suspending fluids rarely differed by as much as 10%; therefore, similar conclusions were reached whether we compared the final concentrations of the 2 hemidiaphragms or compared the 2 distribution ratios (as recorded here). The standard deviation for pairs of hemidiaphragms shaken in two samples of the same medium was about 5% (6 trials).

Illustrative experiments leading to the selection of a medium (medium No. 1) containing 103 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 20 mM sodium pyruvate (and glycine) are recorded in Tables II and III. During 60 min. of equilibration in this medium, the distribution ratio for glycine increased up to 30% over the original value in the rat. If the medium was replaced hourly, the distribution ratio did not fall

TABLE II

Changes of the Distribution of Glycine when the Diaphragm was Suspended in Various Solutions

One hemidiaphragm was analyzed immediately and the ratio of its glycine concentration to that of the plasma (× 1.05) calculated. The other hemidiaphragm was analyzed after 60 min. in the indicated medium, and its glycine referred to that of the suspending medium.

Animal no.	Medium	Plasma glycine N	Fluid glycine	Diaphragm *in vivo*	Glycine *in vitro*	Per cent change of distribution ratio
		mg.-% (×1.05)	*mg.-%*	*mg.-%*	*mg.-%*	
34	Rat plasma	0.42	0.52	1.90	1.78	−29
35	Krebs-Ringer-bicarbonate solution (KRB)	0.55	0.53	2.4	1.8	−22
33	Same, but with 80 meq. K/l.	0.38	0.71	2.1	1.50	−62
39	NaCl 129 meq/l. $NaHCO_3$, 25 meq/l.	0.59	0.62	3.0	1.7	−45
45	KRB+KCN, 3 mM.	0.47	0.66	2.03	1.4	−51
44	KRB+sodium pyruvate, 20 mM.	0.34	0.37	1.50	1.80	+ 4
51	KRB+sodium citrate, 15 mM.	0.46	0.46	2.13	1.56	−26
54	KRB+pyruvate, less KCl and KH_2PO_4	0.65	0.42	2.30	1.91	+31
62	Medium 1[a], 20 meq. Ca^{++}/l.	0.36	0.41	1.70	1.60	−20
90	Medium 1, 180 min. equilibration, renewed hourly	0.63	0.43	2.04	1.59	+14
63	Medium 1. 0.75% glucose replacing pyruvate	0.41	0.44	1.83	1.40	−29
58	Medium 1+DNP, 10^{-4} M	0.24	0.52	1.28	1.11	−60
64	Medium 1+DNP, 10^{-5} M.	0.40	0.46	2.13	1.67	−32
56	Rat plasma+pyruvate, 20 mM.	0.36	0.32	1.33	1.39	+16

[a] NaCl 103, $NaHCO_3$ 25, $CaCl_2$ 5, and sodium pyruvate 20 meq./l.

TABLE III

Changes in the Distribution Ratios of Glycine ([Diaphragm]/[Suspending Fluid]) Resulting from Modification of the Suspending Fluid

One hemidiaphragm was equilibrated 60 minutes in the usual control medium, the other simultaneously in this medium modified as indicated. Where electrolytes were added the sodium chloride content of the medium was correspondingly decreased.

Animal no.	Modification	Per cent change in distribution ratio
80	Succinate replacing pyruvate	−13
89	Succinate replacing pyruvate	−14
85	α-Ketoglutarate replacing pyruvate	+ 9
87	α-Ketoglutarate replacing pyruvate	− 3
145	Fumarate replacing pyruvate	−17
146	+ 20 mM fumarate	−14
127	Additional pyruvate, total 40 mM/l.	−15
148	+ ATP, 10 mM[a]	−23
93	Higher pH, $(HCO_3^-) = 35$ mM	+11
96	Higher pH, $(HCO_3^-) = 50$ mM	− 8
159	+ 10 mM KCl	0
128	+1.2 mM NaH_2PO_4	+12
133	+ 1.2 mM $MgSO_4$	+ 6
135	+ both NaH_2PO_4 and $MgSO_4$	+ 8
78	+ 3 mM malonate	+10
79	+ 20 mM malonate	−13
92	+ 33 mM malonate	+ 6
119	+ 55 mM malonate	−11
88	+ sodium arsenite 10 mM	−10
91	+ sodium arsenite 25 mM	−56
102	+ dinitrophenol 2×10^{-5} M	−22
97	+ dinitrophenol 1×10^{-5} M	−16
94	+ dinitrophenol 5×10^{-6} M	−10
137	+ iodoacetate, 1 mM	−10
139	+ aminopterin, 0.8 mg./ml.	+ 4

[a] Neither experimental nor control medium contained pyruvate.

appreciably below the original value during 3 hr. This medium was equally effective for the diaphragm of the weanling guinea pig. Replacement of pyruvate by citrate (15 mM/l.) diminished glycine retention, probably because of the binding of calcium ions. ATP (10 mM) was inhibitory (Table III), perhaps for the same reason. α-Ketoglutarate replaced pyruvate; succinate replaced pyruvate but less effectively. Replacement of half the sodium of the medium by potassium led to about as rapid loss of glycine from the diaphragm as under any circumstances observed.

When rat plasma was used as the suspending medium, the concentration of *α-amino acids* (measured manometrically by ninhydrin) in the diaphragm was maintained less successfully than for glycine, the distribution ratio falling, as an average, 52%. The concentration of glycine (Table II), and of the amino acids collectively, was improved by the addition of 20 mM/l. of sodium pyruvate to the plasma. The average fall in distribution ration of α-amino nitrogen in 1 hr. from the value in the intact animal was 35% in the presence of pyruvate.

Effects of Certain Poisons

In a N_2-CO_2 atmosphere, or in a medium containing 3 mM cyanide (Table II), glycine loss from the diaphragm was rapid, being faster in the absence of oxygen. Arsenite at 25 mM concentration had a similar effect (Table III). A surprising resistance to malonate was encountered. After tests at lower levels, all of the NaCl of the medium was replaced by sodium malonate (55 mM). The distribution ratio after 1 hr. was still only 11% lower than with the control medium.

2,4-Dinitrophenol at 10^{-4} M led to a very rapid loss of glycine (Tables II and III). A strong inhibition was still evident at 10^{-5} M, with no effect apparent at 10^{-6} M. Adding dinitrophenol to plasma containing 20 mM/l. of pyruvate inhibited the retention of α-amino nitrogen by the suspended diaphragm, the distribution ratios falling by 52% as an average.

Male rats showed higher plasma glycine concentrations than females, 44 males averaging 0.48 mg.-% of glycine nitrogen, standard deviation 0.08, 46 females 0.35 mg.-%, standard deviation 0.06.

Diaphragm of the Scorbutic Guinea Pig

We had hoped to be able to study *in vitro* the depression of the level of free glycine in the muscle in the scorbutic guinea pig (8). The diaphragm, however, did not share this decrease with skeletal muscle. Diaphragms of either normal or scorbutic guinea pigs (Table IV) were highly responsive to elevations of the extracellular glycine, whether in the intact animal or in the artificial medium. When the glycine concentration of the suspending fluid was double that of the plasma, the glycine content of the diaphragm was almost doubled in 2 hours.

TABLE IV

Responsiveness of Diaphragm of Scorbutic Guinea Pigs to Elevations of Extracellular Glycine

In the first 5 experiments one hemidiaphragm was suspended for 2 hr. in the artificial medium. In the other 2 cases, 20 mM of glycine/kg. body weight was fed, in 3 doses an hour apart (2). The distribution ratios given in this table are the calculated ratios cellular concentration/extracellular concentration (6). Concentrations are in mg.-%. Animals 109 and 111 were normal animals.

Expt.	Pretest glycine N			Glycine N, after exposure to high glycine		
	Fluid	Diaphragm	Dist. ratio	Fluid	Diaphragm	Dist. ratio
109	0.86	2.86	5.1	1.77	4.03	3.4
111	1.10	2.66	3.6	0.96	2.41	3.7
112	0.98	2.66	4.1	1.80	4.68	3.9
113	0.41	1.45	5.5	1.20	2.80	3.5
114	0.78	1.92	3.7	1.16	2.72	3.2
115				11.1	14.0	1.8
116				10.0	11.7	1.6

Temperature Effects

Pairs of rat hemidiaphragms were shaken in an initially glycine-free medium No. 1, one of the hemidiaphragms at 37°C., the other at 1°C. The "cold" diaphragm lost half as much glycine nitrogen in an hour as the "warm" one (*e. g.*, 0.65 mg.-% compared with 1.30 mg.-%, as determined by the glycine found in the fluids). A coefficient of approximately 2 for this 36° temperature difference is compatible with the view that the leakage of glycine from the tissue into a glycine-low fluid was a simple diffusion process. Subsequently the "warm" diaphragm was restored to a medium of glycine concentration similar to that of the plasma. Reassimilation against the gradient occurred in 2 hr. to approach the original distribution ratio (*e. g.*, 4.2, compared with the ratio of 4.7 in the intact rat).

The temperature coefficient of the *uptake* of glycine was studied by placing one hemidiaphragm in the usual medium containing about 1 mg.-% of glycine nitrogen, which is about twice the plasma concentration, and equilibrating at 1°, 20°, 30° or 38°C. The other hemidiaphragm and the plasma were extracted for analysis at once after sacrifice of the rat. The net transfer of glycine nitrogen to the diaphragm (*i. e.*, mg.-% after equilibration minus mg.-% in the intact animal) is

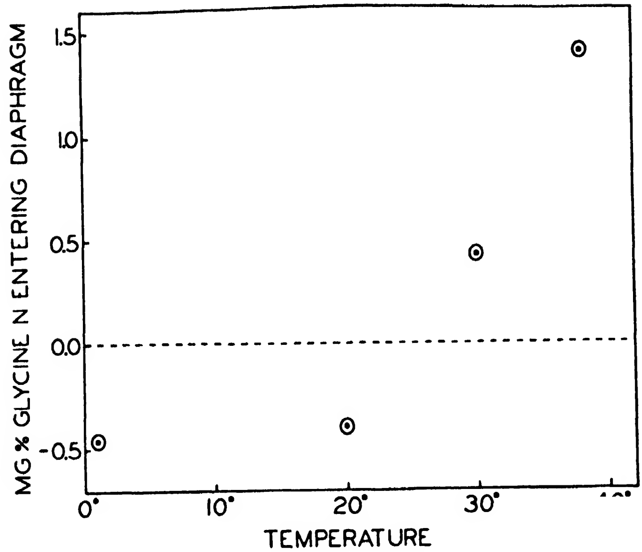

FIG. 1. The change in the concentration of glycine nitrogen of the diaphragm, in 2 hr. in a medium containing about 1 mg.-% of glycine nitrogen, plotted against the temperature. The glycine nitrogen concentrations found, in mg. per 100 g. water, for the plasma and for the artificial medium at the end of the experiment were, respectively: for the experiment at 1°, 0.50 and 0.90; at 20°, 0.48 and 1.00; at 30°, 0.53 and 0.97; at 38°, 0.53 and 1.06.

shown in Fig. 1. Between 30° and 20°C. a reversal occurred in the direction of glycine transfer. While these experiments do not give us a temperature coefficient for the inward migration of glycine, they do indicate that the concentration process depends upon temperature-sensitive reactions.

DISCUSSION

These experiments appear to show that the high glycine content of the diaphragm is maintained by an active concentrating process. The diaphragm glycine level was responsive to the fluid concentration, falling when placed in a medium of one glycine concentration and rising in a medium of slightly higher concentration. Glycine could be eliminated from and then restored to the diaphragm by manipulating the level of glycine in the fluid.

The stimulating effect of pyruvate and α-ketoglutarate, and the inhibiting effect of several factors (anoxia, low temperature, presence of cyanide and particularly of 2,4-dinitrophenol (9)) bear upon the

question of the energy source for the concentrative assimilation of amino acids. Our observations on the distribution of α-amino nitrogen indicate that our conclusions are not limited to glycine but may apply for other amino acids.

Gale (10) has found that gram-positive bacteria also require exergonic metabolism to concentrate certain amino acids from the suspending fluid. Strains of *Strep. faecalis* do not establish equilibrium with a new environment either by *gaining* or by *losing* cellular glutamic acid, unless glucose or another appropriate energy source is present. Cells of *S. aureus*, in contrast (and more like the rat diaphragm) show a slow leakage of glutamic acid in the absence of glucose, which is checked rather than enhanced by the addition of glucose. Brain slices likewise appear to require energy-yielding metabolic reactions in order to assimilate glutamic acid (3).

Human erythrocytes, with their low metabolic activity, contain α-amino acids (collectively by ninhydrin, correcting for glutathione) only 1.2–1.5 times as concentrated in the cell water as in the plasma water (11); glycine was concentrated about 1.7 times, alanine little, if at all (12). Similarly, the rate of entrance of these amino acids into erythrocytes relative to other tissues is extremely slow (12).

Summary

1. The distribution of glycine and of α-amino acid nitrogen between excised diaphragm and artificial suspending medium has been investigated.

2. In a NaCl-$NaHCO_3$ medium containing Ca^{++} ions and sodium pyruvate, the distribution of glycine between diaphragm and fluid was maintained at distribution ratios as high as in the intact animal for 1–3 hr. If the medium was richer in glycine than the plasma, glycine entered the diaphragm against the concentration gradient; at lower fluid concentrations glycine moved from the tissue to the medium.

3. The concentrating activity was strongly inhibited by the following agents and conditions: anoxia, cyanide, 2,4-dinitrophenol at very low concentrations, potassium ion, and arsenite. α-Ketoglutarate and succinate were active in replacing pyruvate. Malonate had comparatively little effect. These observations bear upon the energy source for the concentration of amino acids.

4. The stimulating effect of pyruvate and the inhibiting effect of

dinitrophenol were confirmed for the α-amino acids collectively as measured manometrically by ninhydrin.

5. Male rats were observed to have about one-third higher plasma glycine levels than females.

6. The diaphragm of the guinea pig, whether scorbutic or normal, was similarly responsive as to its glycine content to the glycine level of the suspending medium.

7. The temperature coefficient of the outward migration of glycine from a diaphragm placed in a glycine-free medium was compatible with the process of simple diffusion. The direction in which glycine moved in a glycine-rich medium was reversed from inward to outward by lowering the temperature, indicating that the steepness of the gradient was dependent upon temperature-sensitive reactions.

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Concentration of Amino Acids by the Excised Diaphragm Suspended in Artificial Media. II. Inhibition of the Concentration of Glycine by Amino Acids and Related Substances

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Introduction

Whenever the amino acid concentration of the plasma was elevated by the feeding of any of a number of amino acids to the guinea pig, the free glycine content of the liver or muscle was decreased, or that of the plasma increased; or, as we interpreted the observation, the extent to which these tissues *concentrated* glycine was diminished (1). The magnitude of this effect was roughly proportional to the elevation of the plasma amino nitrogen, no matter which of several amino acids was fed. Conversely, high plasma glycine levels diminished the "concentration" of non-glycine amino acids. This inhibition of the concentrating of one amino acid by another was interpreted as a competition based upon structural similarities. This type of inhibition has now been observed and studied using the isolated rat diaphragm, suspended in an artificial medium (2).

Experimental

Paired hemidiaphragms were shaken for 3 hr. at 37°C. in separate flasks containing 2–2.2 ml. of the artificial medium (NaCl 103 m*M*, $NaHCO_3$ 25 m*M*, $CaCl_2$ 2.5 m*M*, sodium pyruvate 20 m*M*, in an atmosphere of oxygen containing 5% CO_2). To the medium used for one hemidiaphragm the amino acid was added at a concentration of 21.4 m*M*. The media were replaced hourly. In the cases where the amino acid had a net charge at pH 7.4 (*e.g.*, sodium glutamate, arginine monohydrochloride) the NaCl of the experimental medium was correspondingly diminished by 21 m*M*/l. Glycine was incorporated into all media at a concentration estimated to be similar to

that of the plasma, *i.e.*, about 0.48 mg.-% of glycine nitrogen for male rats, about 0.35 for females. During the first and second hours of incubation there was a barely measurable augmentation of the glycine concentration of the 2 successive portions of media supporting the experimental hemidiaphragm, relative to the media for the control hemidiaphragm due to loss of glycine from the tissue. The 2 final solutions serving for the third hour were analytically identical in their final glycine concentrations. Therefore, it was necessary to compare only the final glycine concentrations of the 2 hemidiaphragms. The glycine concentrations of the plasma and the 2 final media were determined to confirm the similarity of the glycine concentration of these three. Other experimental and analytical details have been described previously (2).

Effect of Excesses of Various Amino Acids upon glycine Determination

Three of the amino acids added in excess (20–60 moles/mole of glycine) interfered so strongly with the glycine analyses as to preclude their study, unless they were to be removed before glycine analyses. Histidine and tryptophan prevented good recoveries of the formaldehyde resulting from the reaction of glycine and ninhydrin. Alexander, Landwehr and Seligman (3) suggested that such effects were due to combination of formaldehyde with amino acids. Phenylalanine in excess (30 moles/mole of glycine) interfered by giving a volatile product, presumably phenylacetaldehyde, which halved color formation and, in larger excesses, led to turbidity in the color reaction of formaldehyde with chromotropic acid. These interferences depended upon the presence of large excesses of the amino acids. β-Phenylglycine did not interfere.

Several other amino acids (*e.g.*, aspartic and glutamic acids, proline, ornithine) diminished the recoveries of glycine by 10 or 15% when present in excesses of 60 moles/mole of glycine. In the proportions actually found present in diaphragms at the end of an equilibration (*e. g.*, 7:1, 5:1), the interference was not measurable. By doubling the ninhydrin taken (using 2% rather than 1% ninhydrin solution), the interference was decreased. Other amino acids studied (*e.g.*, leucine, valine, serine) were without effect upon the glycine analyses at excesses of 60 moles/mole of glycine. Recently, Krueger (4) has reported interferences by a number of amino acids with the method of Alexander *et al.* for the determination of glycine. These losses are, for the most part, far larger than we have encountered. The only suggestion that we can make to account for these differences is the empirical and highly critical nature of the conditions required for the distillation. In agreement with our results, Kreuger found that increasing the excess of ninhydrin diminished the interference.

Because glycine is one of the more abundant of free amino acids in the diaphragm (about one mole out of every 8, excluding glutamine) interference by such amino acids as histidine and tryptophan normally present in diaphragm should not be serious, although the possibility of such losses exists. The levels of these free amino acids in the diaphragm are not known. Added glycine could, however, be recovered satisfactorily from diaphragm filtrates.

RESULTS

The consequences of including aminó acids and other substances structurally related to glycine in the medium are illustrated in Table I.

TABLE I

Changes in the Glycine Concentration of Diaphragm Resulting from the Presence of Amino Acids and Related Substances in the Suspending Fluid

One hemidiaphragm of a rat was equilibrated in the control medium, the other simultaneously in a medium identical except for the presence of the amino acid at 21.4 m*M* concentration. After 3 hr., the media being renewed hourly, the tissue samples and final fluid media were analyzed. The values given are the percentages by which the glycine contents of the experimental hemidiaphragms were less than the glycine contents of the control samples. Since the glycine contents of the two media were practically identical at the end of each experiment, the values given correspond also with the decrease of the distribution ratios.

Substance	Decrease in glycine concentration	Substance	Decrease in glycine concentration
	per cent		*per cent*
DL-Serine	−37	D-Leucine *vs.* L-leucine	0
L-Valine[a]	−30	DL-Aspartic *vs.* L-aspartic	0
L-Proline	−35	DL-α-Amino-α-methylbutyric acid	−20
L-Leucine	−25	α-Aminoisobutyric acid	−24
L-Aspartic acid	−35	Betaine	−15
L-Asparagine	−33	Glycocyamine	−14
L-Glutamic acid	−21, −16	Creatine	0, −3
L-Glutamine	−18	β-Alanine	−3
L-Ornithine	−20	DL-β-Aminobutyric acid	0
L-Arginine	−28	*p*-Aminobenzoic acid	−4
10 Amino acids[b]	−29	N-Formyl-L-valine[d]	−1
7 Amino acids[c] *vs.* L-leucine	−17	Niacinamide	−2

[a] From Dr. R. C. Corley, Purdue University.

[b] L-Aspartic, L-glutamic, L-asparagine, L-glutamine, L-arginine, L-ornithine, L-valine, L-leucine, DL-serine and L-proline, each 2.1 m*M*.

[c] DL-Aspartic, L-asparagine, L-arginine, DL-valine, L-leucine, DL-serine and L-proline, each 3.0 m*M*. The control medium contained L-leucine, 21.4 m*M*.

[d] Gift of Dr. J. W. Hinman, Research Laboratories, The Upjohn Company.

All α-amino acids studied were inhibitory. Seven α-amino acids diminished the glycine retention of diaphragm by 25–37%. The effect of glutamine and glutamic acid appeared to be smaller, 16–21%. When L-leucine and D-leucine were added to the suspending media for each of a pair of hemidiaphragms, the final glycine contents of the two hemidiaphragms were identical. The same was true for L-aspartic acid and DL-aspartic acid. Three amino acids in which the amino group was not

in the α position (β-alanine, DL-β-aminobutyric acid, *p*-aminobenzoic acid) had no significant effect upon the glycine distribution. Two α-methyl-α-amino acids also showed inhibiting effects. Introduction of an N-formyl group into valine abolished its inhibitory action. Betaine and glycocyamine were somewhat inhibitory to glycine concentration, but under the present conditions creatine did not appear so. Inhibition occurred whether the α-amino acid concentration was brought to 21 m*M* using a single amino acid or a mixture of 7 or 10 amino acids.

Discussion

The plausibility of attributing the inhibition of the type observed to metabolic products of the amino acid rather than to the amino acid itself is much less with the isolated diaphragm. This is particularly true in the case of the two α-methyl-α-amino acids, which are degraded little if at all by the dog (5). The present technique did not permit us to examine for the criterion of the competitive nature of the inhibition, since we could vary the glycine concentration only narrowly without stimulating excessive transfers of glycine. Several inferences as to the structural requirements for competition may be made:

1. The size and charge of the side-chain did not appear to be important in determining the degree of inhibition, as if the differentiation between glycine and other amino acids were simply on the basis of the absence or presence of a side-chain.
2. Competition did not occur when the amino group was not in the α position. Similar results were obtained upon feeding amino acids to guinea pigs.
3. The presence of N-alkyl groups did not necessarily eliminate competition (*e.g.*, proline, betaine, glycocyamine).
4. N-Acylation, with loss of the positive charge, eliminated inhibition in the case of formylvaline.
5. D- or L-amino acids appeared to be equally effective. This is perhaps not surprising in view of the absence of optical asymmetry in glycine.
6. A high α-amino acid concentration, rather than an extreme concentration of a single amino acid, appeared responsible for the inhibition of glycine retention.

The probable relation of the loss of amino acids from the interior of

the cell, as a result of the process reported here, to the nutritional disadvantage of diets containing excessive quantitites of one or several amino acids has already been discussed (1). Certain amino acid mixtures developed recently can be infused intravenously with extreme rapidity into patients, producing very high plasma amino acid concentrations, but no nausea. Until the nutritional consequences of such high amino acid levels (which do not represent all amino acids) are known, it is suggested that the convenience of rapid infusion may be overemphasized.

Summary

Each of a group of α-amino acids inhibited, at a 21 mM concentration the concentrating of glycine by the excised diaphragm suspended in an artificial medium. The effect was obtained only when the amino group was in the α position. An N-formyl group abolished the effect of valine. N-alkyl groups did not necessarily eliminate the inhibition. No optical specificity was observed. These effects are related to similar inhibitions observed in the intact animal.

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Substrate Concentration and Specificity of Choline Ester-Splitting Enzymes [1]

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Introduction

For the assumption that the release and removal of acetylcholine are events essential for conduction as proposed by Nachmansohn (1), it is of interest to know how far the esterase present in conductive tissue is specific for acetylcholine and distinguishable from other esterases. Stedman, Stedman and Easson (2) and Simonart (3) found that propionylcholine and butyrylcholine are split by serum esterase at a higher rate than acetylcholine. Their findings, confirmed by Glick (4), do not support the assumption that the serum esterase is an enzyme specific for acetylcholine. Nachmansohn and Rothenberg (5) demonstrated that, in striking contrast to serum esterase and many other esterases, the esterase in a great variety of conductive tissue, nerve, and muscle, hydrolyzes propionylcholine at a lower rate than acetylcholine or, in a few cases, at the same rate, whereas butyrylcholine is split at a very low rate or not at all. Only red blood cell esterase showed the same pattern of hydrolysis rates when different substrates were used. Nachmansohn and Rothenberg came to the conclusion that the esterases in conductive tissue have a high, though not absolute, specificity for acetylcholine, since no other ester tested is split at a higher rate.

A second difference by which the choline ester-splitting enzymes may be distinguished, is the effect of substrate concentration. As was shown by Alles and Hawes (6), there is a definite optimum concentration of acetylcholine for red blood cell esterase. Excess of the ester strongly inhibits the enzyme. This finding has been confirmed and extended to the esterases of brain (7) and many other conductive tissues (5,19). However, the relationship between activity and substrate concentration may differ for a given enzyme from substrate to substrate. The importance of a careful consideration of the substrate concentration in following the enzymic hydrolysis of various esters has been recently demonstrated by Augustinsson (8,9). The erythrocyte esterase splits, *e. g.*, acetyl-β-methylcholine more slowly than acetylcholine at low substrate concentration but more rapidly when the substrate concentrations are high. This

[1] The work has been carried out under a grant from the U. S. Public Health Service.

[2] Fellow of the American-Scandinavian Foundation, with additional grants from the Swedish National Medical Research Council and the U. S. Public Health Service.
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shows that, although both esters inhibit the enzyme in high concentration, the optimum substrate concentration is not the same for the two esters.

Bodansky (10) reported that the brain and erythrocyte cholinesterases split triacetin, human brain esterase more rapidly than acetylcholine. To clear up this problem and other conflicting results by various investigators regarding the specificity of choline ester-splitting enzymes, the following study has been performed.

METHODS

The esterase activity was measured by the Warburg manometric method. In modification of the usual technique, 1.60 ml. of the substrate solution was placed in the main compartment of the vessel and 0.40 ml. of the enzyme solution in the side bulb (8). Substrates and enzyme preparations were dissolved in a bicarbonate-buffer solution containing 0.15 M NaCl, 0.04 M $MgCl_2$ and 0.025 M $NaHCO_3$ (5). The hydrolysis was carried out in a gas mixture of 95% N_2–5% CO_2. After attaining temperature equilibrium, the first manometer was red and the contents of the other flasks were mixed at zero time. At 1 min. intervals, the contents of the other flasks were mixed. Each manometer was red at 6 min. intervals, 1 min. between each manometer reading. Readings were made continuously for 36 min. Measurements were made at 23–24°C.

The output of CO_2 expressed in μl. was plotted against time. The interpolated 30 min. value, minus the amount of CO_2 evolved during the same time period by nonenzymic (spontaneous) hydrolysis, evaluated in each case, has been used as unit (b_{30}) in expressing the esterase activity.

The tissue was homogenized according to the technique described by Potter and Elvehjem (11). Aliquot parts of the homogenized tissue or of the supernatant fluid obtained after centrifugation were then used as enzyme preparations.

Fresh solutions of the substrates (in bicarbonate-buffer solution) were made for each experiment. The esterase activity was measured at 6 various substrate concentrations. The final percentage concentrations of the substrates after mixing with the enzyme solutions were: 2.00, 0.60, 0.20, 0.060, 0.020, and 0.006.

The spontaneous hydrolysis of the substrates at various concentrations was observed for each substrate. The enzymic activity, expressed by b_{30}, was plotted against pS, the negative logarithm of molar substrate concentration.

Selection of Material

For the study of enzyme kinetics in general the ideal condition would be the use of pure enzymes. Such preparations, however, are not readily available. For the present study, two highly purified preparations were available in which the enzyme may be considered as nearly pure: esterase from human serum and from electric tissue of *Electrophorus electricus*.[3] The first was used as an example of a choline ester-splitting

[3] I am greatly obliged to Dr. A. Goldstein, Harvard Medical School, for the preparation of the serum esterase (Dr. E. Cohn's fraction IV-6-3), and to Dr. M. A. Rothenberg, of this laboratory, for the electric tissue esterase.

enzyme, which, on the basis of previous experiments, does not split acetylcholine at the highest rate; the second as an example of an esterase of conductive tissue assumed to have the highest affinity to acetylcholine.

As pure enzyme preparations from other tissues were not available, those conductive tissues were selected which contain a high concentration of the enzyme, so that relatively small amounts of tissue could be used. In this way, the probability of interference by other enzymes or proteins becomes smaller. Examples of 4 types of conductive tissue were chosen: nerve and muscle tissue of vertebrate and invertebrate animals. One of the highest concentrations of the choline ester-splitting enzyme in mammalian nerve tissue has been found in the nucleus caudatus, and in the invertebrate nerve tissue in the head ganglion of squid. Both were found to be relatively specific for acetylcholine (5). In addition to the figures published previously, new data concerning the activity and relative specificity of esterases in conductive tissue are summarized in Table I.[4] On the basis of these data, *Lebistes* muscle was chosen as

TABLE I

Rate of Hydrolysis of Acetylcholine (ACh), Propionylcholine (PrCh) and Butyrylcholine (BuCh), and Triacetin (TA) by Various Conductive Tissue at Optimum Acetylcholine Concentration

Tissue	Animal	ACh split mg./g./hr.	PrCh	BuCh	TA
			In per cent of ACh hydrolysis		
Brain	Pigeon	240	80	0	5
	Sparrow	300–350		5	
	Humming bird	300		0	
	Turtle	80–100	70	0	4
	Frog	60	50	0	15
Muscle	*Lebistes*	300–400	75	12	10
	Lizard	40		3	
	Nereis	60–65			20
	Lumbricus	140–160	70	15	10

vertebrate and the body wall of *Lumbricus* as invertebrate muscle.

Erthrocyte esterase has been tested since it is known to be similar to nerve and muscle esterase (5,8). The blood of *Helix aspersa* has also been studied, since it has been found that the cell-free blood of *Helix pomatia* is similar to the nerve and erythrocyte esterase, although different in a few respects (8,9). The dart sac of *Helix pomatia* has a very high esterase activity (8,9). It appeared desirable to obtain additional information regarding some peculiar properties of this enzyme.

[4] These determinations were carried out by Mrs. Claire Marshall and Mrs. Emily Feld-Hedal in Dr. Nachmansohn's Laboratory and the unpublished data were kindly put at my disposal.

Snake venom from *Colubridae* has a very high cholinesterase activity. Zeller (12) assumes that the snake venom cholinesterase is different from his "s" type and "e" type cholinesterases and classifies it as a new "c" type (colubercholinesterase).

Results

1. Purified Esterase Prepared from Human Blood Serum

The results obtained with the highly purified esterase from human blood plasma are presented in Fig. 1. The dissociation curve for acetylcholine is the same as that obtained with the partly purified preparation from horse serum described previously; the pK_s is 2.5 (8). Propionyl-

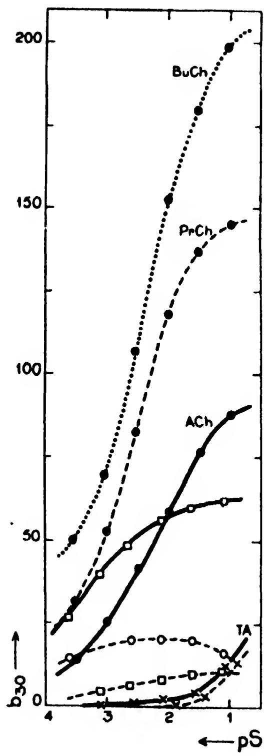
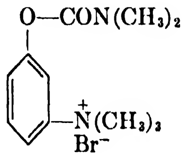


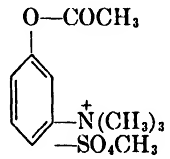
FIG. 1. Activity-*pS* curves for the enzymic hydrolysis of various esters by a purified esterase preparation from human blood serum (Fraction IV-6-3 according to Cohn (18)). 0.8 mg. enzyme per vessel. ● ———— ●, Acetylcholine chloride (ACh); ● - - - - - - - - ●, Propionylcholine chloride (PrCh); ● ·········· ●, Butyrylcholine chloride (BuCh); ○———————○, *dl*-Acetyl-β-methylcholine chloride; ○- - - - -○, Benzoylcholine chloride; ×——————×, Triacetin (TA); ×- - - - - - - -×, Methyl butyrate; □——————□, Nu 2017; □- - - - - - - -□, Nu 2416.

choline is split at a higher rate than acetylcholine and butyrylcholine at a still higher rate, confirming earlier findings. Both these esters give the same value of pK_s as acetylcholine. The hydrolysis of benzoylcholine is depressed by excess of substrate. The optimum substrate concentration is at a pS of 2, which is again in agreement with the previous observation. Methyl butyrate, tributyrin and triacetin are split very slowly. The affinities of the noncholine esters are definitely lower than that of the choline esters.

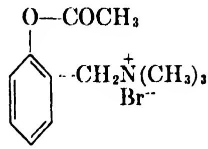
Two other compounds similar in structure to prostigmine were tested: 3-acetoxyphenyl trimethylammonium methylsulfate (Nu 2017) and 2-acetoxybenzyl trimethylammonium bromide (Nu 2416).[5]



Prostigmine bromide



Nu 2017



Nu 2416

Nu 2017 has a relatively high affinity for this esterase (pK_s 3.7). Nu 2416, on the other hand, is split at a very low rate. The differences in pK_s for the hydrolysis of acetylcholine and Nu 2017 may explain why acetylcholine is split at a lower rate than Nu 2017 at low substrate concentration, at a higher rate when the concentrations are higher. The affinity of Nu 2017 for the enzyme is higher, but the hydrolysis rate of the enzyme-substrate complex is lower than for acetylcholine. The low value of K_s in the case of Nu 2017 is consistent with the high affinity of prostigmine for the enzyme.

The spontaneous hydrolysis of Nu 2017 and Nu 2416 is more rapid than that of acetylcholine. At 23°C., the amounts of CO_2 in µl. evolved during 30 min. from 2.0 ml. of 0.025 *M* bicarbonate solution are the following:

Molar conc.	Acetylcholine chloride	Nu 2017	Nu 2416
1×10^{-1}	8	18	14
3×10^{-2}	5	8.5	4
1×10^{-2}	3	5	1
3×10^{-3}	2	4	0
1×10^{-3}	1	3	0

[5] I wish to express my thanks to Dr. J. A. Aeschlimann of Hoffmann-LaRoche, Inc., Nutley, New Jersey, for supplying these compounds.

2. *Purified Esterase Prepared from Electric Tissue of Electrophorus electricus*

The results obtained with the crude preparation and the highly purified solution from the electric tissue of *Electrophorus electricus* are presented in Fig. 2. The activity substrate concentration relationship has been studied for the hydrolysis of various esters. In previous experiments, the substrate concentration was varied only for the acetylcholine hydrolysis, whereas the other substrates were tested only at concentrations close to the optimum of acetylcholine hydrolysis.

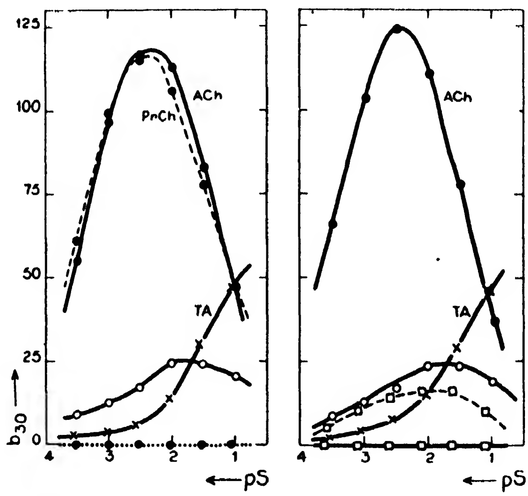


FIG. 2. Activity-*pS* curves for the enzymic hydrolysis of various esters by the electric organ of *Electrophorus electricus*. Left: crude extract; right: purified preparation (1 mg. of protein splitting 20 g. of ACh/hr.). Symbols as in Fig. 1.

Propionylcholine is split at about the same rate as acetylcholine, and butyrylcholine not at all. The patterns are the same for the crude and highly purified preparation and confirm the previous finding (5) that the properties of the esterase of homogenized tissue are unchanged after purification and that acetylcholine at high concentration depresses the enzyme activity. The optimum acetylcholine concentration is 3×10^{-3} *M*. The curves are symmetrically bell-shaped, consistent with Haldane's hypothesis that the formation of an ES_2 complex incapable of breaking down at high substrate concentration may be valid for the hydrolysis of acetylcholine (13). The optimum substrate concentrations are the same for acetyl- and propionylcholine. The contrast to the

serum cholinesterase is striking. Acetyl-β-methylcholine is split at a much lower rate. The optimum substrate concentration (pS_{opt} 1.6) is higher than in the case of acetylcholine and propionylcholine, and the same as for the erythrocyte and brain cholinesterase (8).

The enzyme splits triacetin, but the activity substrate concentration relationships for the hydrolysis of acetylcholine and triacetin are entirely different. At high substrate concentration triacetin is split at a higher rate than acetylcholine; at low substrate concentrations, triacetin is split at a much lower rate. The affinity of the enzyme for acetylcholine is much greater than for triacetin.

The effect of the esterase of electric tissue on the synthetic substrates also differs from that produced by the serum esterase; Nu 2416 is split slowly and Nu 2017 not at all.

3. Mammalian Nerve Tissue (Nucleus Caudatus of Ox)

In Fig. 3 are shown the results with an esterase obtained from mammalian brain tissue (nucleus caudatus of ox). The substrates used were acetylcholine, butyrylcholine, and triacetin. The data confirm the previous finding that the enzyme is inhibited by high acetylcholine concentrations. Butyrylcholine is split at a very low rate. The pattern of the activity-pS curves with the 3 substrates used is strikingly similar to that of the highly purified electric tissue esterase.

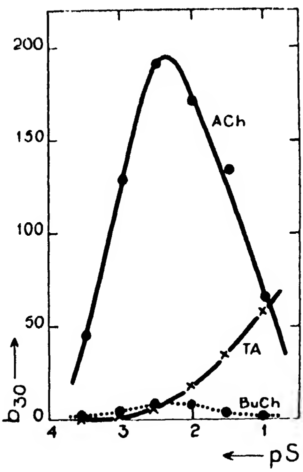


Fig. 3. Activity-pS curves as in Fig. 1 obtained with an extract from nucleus caudatus (ox).

4. Invertebrate Nerve Tissue (Head Ganglion of Squid)

The esterase of the head ganglion of the squid splits acetylcholine at a higher rate than the other substrates tested, confirming the previous statement (5). Propionylcholine is split at a lower rate, and butyrylcholine still more slowly. The activity-substrate concentration relationships are the same as those obtained with the electric

tissue (Fig. 4). As in the case of electric tissue esterase, the optimum substrate concentration for the hydrolysis of acetyl-β-methylcholine (pS_{opt} 1.6) is higher than that for acetylcholine (pS_{opt} 2.5). Therefore, acetyl-β-methylcholine may be split at the same, or even a higher, rate than acetylcholine when the concentrations of the two sub-

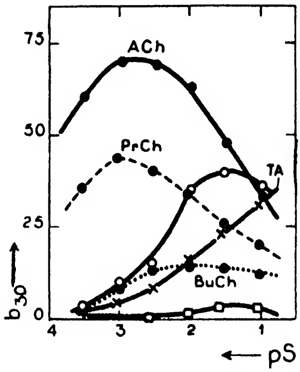


FIG. 4. Activity-pS curves as in Fig. 1 obtained with an extract from squid ganglion.

strates are high, close to 0.1 M. In low concentrations, on the other hand, close to 0.001 M, acetylcholine is split much more rapidly than acetyl-β-methylcholine. These differences apparently explain the findings of Richards and Cutkomp (14) and Tobias and coworkers (15), that insect nerve tissue was more active on acetyl-β-methylcholine than on acetylcholine, since the molar concentrations of the substrates used by these authors were high (0.1 M). Benzoylcholine is not split at all by the squid ganglion esterase.

The effect of substrate concentration on the hydrolysis of triacetin is similar to that found in the electric tissue esterase. The esterase of squid ganglion splits Nu 2017 very slowly.

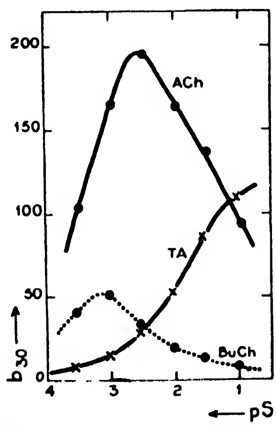


FIG. 5. Activity-pS curves as in Fig. 1 obtained with an extract from *Lebistes* tail muscle.

5. Vertebrate Muscle (Lebistes reticulatus)

The results obtained with extracts prepared from homogenized tail muscle of *Lebistes reticulatus* are shown in Fig. 5. The optimum concentration of acetylcholine is the same as that obtained with the esterases of nerve tissue. Butyrylcholine is split at a very low rate; the optimum concentration for butyrylcholine is slightly lower than for acetylcholine. The rate of hydrolysis of triacetin differs in the same way as with nerve esterase.

6. Invertebrate Muscle (Lumbricus terrestris)

The activity-pS curves for the hydrolysis of different substrates by washed, homogenized *Lumbricus* muscle (Fig. 6) resemble those with vertebrate muscle, but the

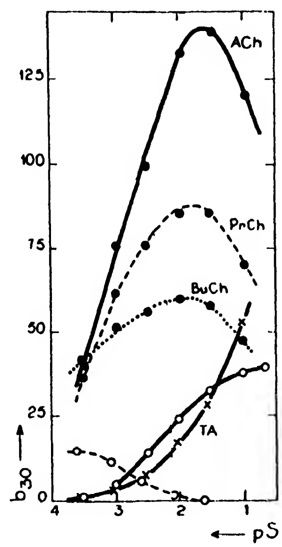


FIG. 6. Activity-pS curves as in Fig. 1 obtained with an extract from *Lumbricus* muscle.

optimum substrate concentration for choline esters is higher (pS_{opt} 1.7). Only benzoylcholine, which is split at a low rate at all concentrations, has an optimum at a lower concentration.

7. Erythrocytes from Human Blood

Human red blood cells were washed 3 times with 0.9% NaCl solution and then hemolyzed with twice the volume of distilled water. The hemolyzate was diluted with 10 volumes of bicarbonate Ringer's solution. This solution was used in the experiments and the results are shown in Fig. 7. The optimum substrate concentration for acetylcholine is 3×10^{-3} M. Propionylcholine is split at a lower rate with approximately the same optimum. Butyrylcholine is split at a very low rate or not at all. At the optimum

acetylcholine concentration, acetylcholine is split 10 times faster than triacetin. In 0.1 M concentration, triacetin is split at a rate which is 2.7 times higher than for acetylcholine.

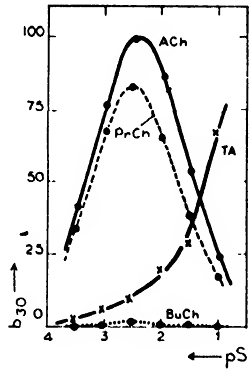


Fig. 7. Activity-pS curves as in Fig. 1 obtained with hemolyzate of human erythrocytes.

The pattern for this esterase shows the similarity to the nerve esterases previously stated (7,5,8). Both enzymes seem to be localized in the surface membrane and certain similarities have been reported concerning the properties of nerve and erythrocyte membranes.

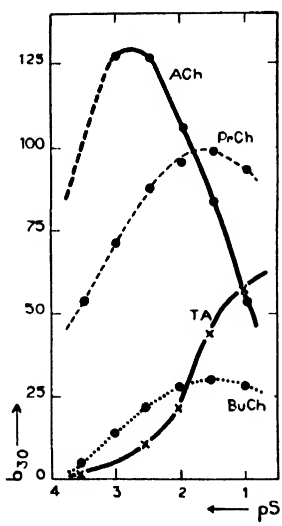


Fig. 8. Activity-pS curves as in Fig. 1 obtained with the blood from *Helix aspersa*.

8. Blood from Helix aspersa

The results obtained with the blood of *Helix aspersa* are shown in Fig. 8. The enzyme displays its optimum activity at pS_{opt} 2.7 when acetylcholine is employed as sub-

strate. In the hydrolysis of propionylcholine, the optimum substrate concentration is higher, about the same as has been previously obtained in the hydrolysis of acetyl-β-methylcholine (pS_{opt} 1.5) (8). This difference in pS_{opt} explains why propionylcholine is split at a higher rate than acetylcholine when the substrate concentrations are high (0.1 *M*). Butyrylcholine is split at a much lower rate and the optimum is the same as for propionylcholine. Triacetin gives the usual dissociation curve. The pattern thus demonstrated for the *Helix* blood is not quite the same as that found for the nerve-muscle-erythrocyte esterases. Differences in other respects have been reported (8).

9. *Dart Sac from Helix aspersa*

It has been pointed out that the dart sac enzyme displays its maximum activity at a higher acetylcholine concentration than is observed with the erythrocyte and brain esterases (9). Fig. 9 confirms this result with the dart sac from *Helix aspersa*. Propionylcholine is split at a low rate and butyrylcholine at a still lower rate. The optimum substrate concentration for all 3 choline esters is 3×10^{-2} *M*, which is 10 times higher than for the nerve and muscle esterases.

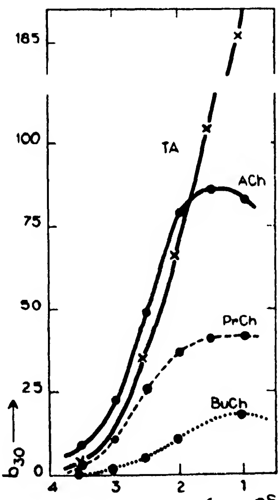


FIG. 9. Activity-*pS* curves as in Fig. 1 obtained with the dart sac from *Helix aspersa*.

Triacetin is split at an extremely high rate in contrast to the findings obtained with all esterases hitherto described. It was assumed that the same enzyme splits acetylcholine and tributyrin (8). However, the very high activity toward triacetin suggests the presence of a second esterase distinct from the acetylcholine-hydrolyzing enzyme. To test this assumption, the rate of hydrolysis was measured in a mixture of acetylcholine and either triacetin or butyrylcholine. The amount of CO_2 evolved was then compared with the amounts when the substrates were hydrolyzed separately. Table II shows the results. Butyrylcholine inhibits the hydrolysis of acetylcholine, which

TABLE II

Rate of Enzymic Hydrolysis of Acetylcholine (ACh), Butyrylcholine (BuCh) and Triacetin (TA), and of Mixtures of these Substrates by the Dart Sac

Rate is expressed as μl. CO_2 evolved in 30 min. (b_{30}). Substrate concentration in each case as follows: ACh: 1.1×10^{-2} *M*; BuCh: 9.5×10^{-3} *M*; TA: 9.2×10^{-3} *M*

Substrate	b_{30}
ACh	80
BuCh	11
TA	67
ACh+BuCh	67.5
ACh+TA	131

indicates that these 2 substrates may compete for the same enzyme. In a mixture of acetylcholine and triacetin, an additive effect is obtained. This apparently indicates that the 2 substrates are split by 2 esterases, acting independently of each other. It is possible that both enzymes split acetylcholine, and this may explain the higher optimum acetylcholine concentration.

It was observed that the dart sac of *Helix aspersa* consists of 2 parts: an outer and an inner tissue. It is possible that one esterase is in the outer, the other esterase in the inner shell, which may be muscular element. Analogous observations have not been reported for *Helix pomatia.*

10. *Snake Venom from Naja naja*

In the present experiments, a dried and crystallized preparation of the venom was used. The activity of this preparation was of the same magnitude as for the electric

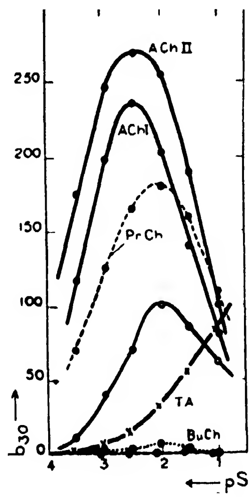


FIG. 10. Activity-*pS* curves as in Fig. 1 obtained with the dried and crystallized venom from *Naja naja*. (ACh I: 0.12 mg. of fresh venom per vessel; ACh II: 0.4 mg of 11 year old venom per vessel.)

organ, on the basis of dry weight. Acetylcholine in high concentration inhibits the activity and the optimum substrate concentration was 3×10^{-3} M, the same as was found for the nerve-muscle-erythrocyte esterases (Fig. 10—ACh I). Propionylcholine is split at a lower rate than acetylcholine, but the optimum substrate concentration is slightly higher and about the same as for acetyl-β-methylcholine; the latter is split at a lower rate. Butyrylcholine and benzoylcholine are practically unaffected. Triacetin gives the usual curve.

Discussion

Two essential results emerge from the experiments reported.

1. Importance of Substrate Concentration. Esterases of the type found in serum show the familiar dissociation curve when the activity is plotted against the log of acetylcholine concentration. This relationship is strikingly different from that found with esterases of conductive tissue and erythrocytes, and of a few special cases like the *Helix* blood and the snake venom. In addition, the various choline esters frequently have different substrate optima. For example, whereas acetylcholine does not inhibit the serum esterase in relatively high concentration (0.1 M), benzoylcholine has a substrate optimum at about $pS = 2$. The activity of the second type of esterase has an optimum for acetyl-β-methylcholine which differs markedly from that for acetylcholine, propionylcholine, and butyrylcholine. Therefore, there is no fixed ratio between the rates of hydrolysis of these 3 esters and that of acetyl-β-methylcholine. Moreover, even for these 3 esters, optimum substrate concentration may not always be the same, as is demonstrated by the results with *Helix* blood esterase.

Considering the hydrolysis of non-choline esters by these esterases, the importance of the activity substrate concentration relationship becomes still more obvious. For instance, triacetin is split by the esterases of the type found in conductive tissue at a low rate at the optimum acetylcholine concentration, but at high substrate concentration, the rate of hydrolysis of triacetin may be even higher than that of acetylcholine. The affinity of such esterases is in all cases much higher for acetylcholine than for triacetin. Testing the hydrolysis rate at that high concentration only gives a distorted picture. One example of this is Bodansky's finding that human brain esterase splits triacetin faster than acetylcholine. Another example is the statement of Mendel, Mundell and Rudney (16) that, in a mixture of esterases, a quantitative distinction may be made between 2 types by the use of acetyl-β-methylcholine and benzoylcholine. The data presented in this paper are incompatible with this view.

It is, at present, impossible to evaluate the physiological significance of the relationship between enzyme activity and substrate concentration. The concentration at which acetylcholine may appear during activity at the site of action is unknown. But is it known that the enzyme is present in an excess of about 10 times, leaving a considerable margin of safety (20). A similar situation may be assumed for substrate concentration, namely, that it usually remains below the optimum; maximum velocity due to optimum concentration may occur only in special conditions, such as pathological disturbances.

2. Specificity of Acetylcholine Hydrolyzing Enzyme. The second important outcome of the data presented is a confirmation and extension of the observations of Nachmansohn and Rothenberg on the specificity of the esterase present in conductive tissues and erythrocytes. The test of the hydrolysis rates of propionylcholine and butyrylcholine appears indeed essential for distinguishing this type of esterase from that present in serum and pancreas. Without the use of these two choline esters, all those enzymes were defined as cholinesterases, which, at optimum conditions, hydrolyze acetylcholine at a higher rate than any other ester (8). Thus, the type of esterases present in conductive tissue—and erythrocytes—did not appear to have sufficiently strong substrate specificity to justify a sharp distinction between this type and other esterases. However, the present study confirms the view of Nachmansohn and Rothenberg that the esterases present in conductive tissue may be distinguished from other choline ester-splitting enzymes by the use of propionylcholine and butyrylcholine.

The patterns of the activity-*pS* curves in nerve and muscle tissue of vertebrate and invertebrate and in the red blood cells are indeed strikingly similar in all essential features. The affinity of acetylcholine to the enzyme is high in contrast to non-choline esters. The bell-shaped curve obtained if the activity is determined as a function of substrate concentration is another distinction and confirms the previous findings of various authors of the inhibitory effect of high acetylcholine concentration on this special enzyme.

All these features support the assumption of the existence of a type of enzyme relatively specific for acetylcholine and distinctly different from other choline ester-splitting enzymes. Therefore, and in view of the rather confusing terminology used by various investigators, Augustinsson and Nachmansohn have proposed for this type of enzyme the term acetylcholine-esterase (ACh-esterase) (17). In addition to the

esterase present in all conductive tissues and erythrocytes, there are 2 special cases in which the esterases appear to have similar features: *Helix* blood and snake venom. The snake venom contains a great variety of enzymes. The hemolytic effect of the venom is well known. It has been recently demonstrated that lysolecithin, which is assumed to be a hemolytic factor, releases the esterase within the red blood cells (8). It is not impossible that red blood cells may be one source, if not the main source, of the effect exerted by the venom. No suggestion can be made as to the presence of the esterase in *Helix* blood. The enzyme has a few features which distinguish it from the ACh-esterase, but more information is necessary before a conclusion is permissible.

The properties of the esterase preparation obtained from the dart sac of *Helix*, which offered some difficulty as to classification (8), have found·a satisfactory explanation from the evidence that the preparation contains two different types of esterases.

Acknowledgments

I wish to express my thanks to Dr. David Nachmansohn for his stimulating interest and his valuable suggestions. I want to thank Mrs. M. Augustinsson for her unfailing assistance in the experimental work.

Summary

Whereas choline ester-splitting enzymes of the type present in serum give the usual dissociation curve, the esterases from all conductive tissue tested and from erythrocytes show a rather sharp optimum concentration of acetylcholine. The optimum substrate concentration is for some choline esters almost the same, but different for others. In contrast, triacetin is split at a low rate in low, and at a high rate in high concentrations.

The experiments illustrate the importance of substrate concentration when the enzyme activity is measured toward different substrates. They support the assumption that the type of esterase present in conductive tissue and erythrocytes, and possibly in a few special cases, has well-defined properties distinctly different from other choline ester-splitting enzymes.

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The Polysaccharide from *Iles mannane*

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Introduction

Various species of *Amorphophallus* from Java are known to contain appreciable amounts of polysaccharidic material. The polysaccharides may be isolated by water extraction of the dried roots. In 1939, a number of such roots (representing various species) were examined by de Groot, van Hulssen and Koolhaas (1), and all were found to contain mannose-yielding carbohydrates in appreciable amounts. Among them was the so-called *Iles mannane* (from *A. oncophyllus*), the polysaccharide of which gave rise to over 50% mannose on hydrolysis. The aqueous solutions of this polysaccharide were extremely viscous, and the carbohydrate could be precipitated from such solutions by means of alcohol. Experiments at The Institute of Paper Chemistry have shown that the *Iles mannane* polysaccharide is an excellent beater additive when used in paper manufacture.

Analytical data, given in detail in the experimental part, show that the carbohydrate consists mainly of glucose and mannose units, with subordinate amounts of pentosans and uronic acid units. Tests for fructose, galactose, and galacturonic acid groups were negative. Thus, the polysaccharide of *Iles mannane* may be a mannoglucan (but the possibility of a mixture of mannan and glucan is not excluded).

Experimental

An orienting experiment was carried out with a small sample of ground powder of *Iles mannane* (also termed *Iles iles*) obtained through the courtesy of the Trade Commissioner of the Netherlands Indies. The powder was sifted into boiling water and the mixture stirred for 10–15 min.. cooled, and centrifuged. The nearly colorless liquid was decanted, and the aqueous solution precipitated by admixture with several volumes of 95% ethanol. The resulting precipitate was obtained both in a gelatinous fibrous form and in a flocculent form. On extensive centrifuging of the aqueous-alco-

holic suspension, the "fibrous" form (which predominated) remained in suspension, whereas the flocculent type formed a compact deposit on the bottom of the centrifuge bottle. The fibrous polysaccharide was best removed by filtration on mercerized broadcloth and could then be dehydrated by suspending successively in alcohol, acetone, and ether, using the broadcloth each time as a filtering medium and squeezing out the solvent. The smaller flocs of polysaccharide could be treated with solvents directly in the centrifuge. In either case, the product was dried at room temperature.

The mixture of fibrous and flocculent material was used for the preliminary hydrolyses. Later, larger samples of purified (fibrous) iles mucilage were isolated by a similar procedure, the principal variant being omission of the acetone trituration—*i. e.*, only ethanol and ether were used in the dehydration. In one experiment, 37 g. of air-dried powdered *Iles mannane* (34.8 g. oven dried) were treated with 4.5 l. of water at 90–95°C. and gave about 14.5 g. of (total) polysaccharide. This must be taken as a minimal figure, inasmuch as complete extraction is very difficult.

The mucilage in aqueous solution evidently gives the same type of borax-gel test as that given by the mannogalactans—*e. g.*, locust bean gum (2). Physically, it also resembles the latter. However, it is chemically very different. The cold suspension of iles mucilage in water gives a deep blue coloration with iodine solution. This is not given by the mannogalactans.

Hydrolysis of the air-dried mucilage with 1% H_2SO_4 at the boiling point of the mixture showed (from a study of the hydrolysis-time curve) that the hydrolysis was virtually complete in 12.5 hr. Thus, 150 mg. of air-dried mucilage (139.7 mg. oven dry) yielded (by the Munson-Walker method) 141 mg. of reducing sugars (calculated as glucose) after 12.5 hr. During the hydrolysis, a small amount of dark residue also formed, but this was not studied further.

The filtered neutralized hydrolyzate contained mannose (identified as the phenylhydrazone, m.p. 194.5–195.5°C.) and the filtrate from a quantitative mannose determination, on heating, yielded a voluminous precipitate *qualitatively* identified as phenylglucosazone, m.p. 207–208°C.

The presence of mannose and glucose in the hydrolyzate of *Iles mannane* was confirmed by the following procedure. The hydrolyzate was neutralized with $BaCO_3$. The filtrate was evaporated to a small volume, treated with alcohol to precipitate barium salts, and filtered. This second filtrate was evaporated to a thin sirup, a drop of which was then subjected to the paper partition chromatographic separation devised by Partridge (5). Typical dark brown glucose and mannose "spots" were obtained (identical in their relative positions with those obtained from known pure sugar samples). In this procedure, the uronic acids were presumably removed as barium salts, and pentosans were present in such small quantities that they failed to register on the paper strip. When hydrolyzed for very brief periods with HCl, the iles mucilage failed to respond to the Seliwanoff test for D-fructose, whereas, under identical conditions, inulin gave a characteristic, deep red pigment, soluble in amyl alcohol. Evidently *fructosans are absent* from the iles gum.

Another 500 mg. sample of the iles mucilage was hydrolyzed with 2% HNO_3 for several hours, and then carried through the mucic acid determination for galactose. At the end of 2 days in the refrigerator, the HNO_3 solution showed a faint cloudiness but no weighable precipitate

was obtained. This indicated the *absence of galactose and galacturonic acid.* The absence of galactose was further confirmed by quantitative differential fermentations (6) of the neutralized iles hydrolyzate. When the hydrolyzate corresponding to 100 mg. of air-dried mucilage was fermented by organism N.R.R.L. No. 379, the Munson-Walker reducing value was 36.2 mg. of Cu_2O. When fermented with organism N.R.R.L. No. 966 in a parallel experiment, the final reducing value was 35.8 mg. of Cu_2O. These values are identical (within the experimental error), and clearly indicate the absence of galactose. As indicated above, the mannose in the H_2SO_4 hydrolyzate was determined quantitatively as the phenylhydrazone. Uronic anhydride was determined by Browning's procedure (3), and pentosans were estimated by Kröber's method (4).

The total Cu_2O Munson-Walker reducing value obtained from 100 mg. of the polysaccharide after hydrolysis corresponded to 101 mg. of sugar calculated as glucose. The Cu_2O reducing values corresponding to the predetermined mannose and uronic anhydride values could be read from the Munson-Walker tables of Wise and McCammon (7) and, for each of these, a corresponding "glucose" value could be calculated. The sum of these calculated values, subtracted from the original 101 mg., gave a measure of the actual glucose in the hydrolyzate. Admittedly, such a calculation is highly proximate, but it serves to show that a substantial portion of the hydrolyzate is D-glucose.

A proximate analysis of the mucilage (oven dry basis) follows:

	Per cent
Mannan	41
Glucan (calculated from Munson-Walker reducing values)	48.6
Uronic anhydride	3.6
Pentosans	1.76
Ash	0.53

The above values for glucan and pentosans are given with reservations, but it is apparent that the mucilage contains largely *mannose* and *glucose groups.* How these exist in the mucilage is problematical. The glucose may emanate from a true mannoglucan or, possibly, from a mixture of mannan and glucan.

Acknowledgments

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The Distribution in Rat Tissues of the Methylene Carbon Atom of Glycine Labeled with C^{14} [1]

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Introduction

In the course of studies concerning the role of glycine as a precursor of hemin (1), data on the distribution of the labeled α-carbon atom of glycine have been gathered. These data are presented in this paper. The metabolism of glycine labeled with N^{15} in the amino group, or with C^{13} and C^{14} in the carboxyl group, has been studied previously by several groups of investigators (2,3,4,5,6). Since, on the basis of these and other studies (7), it seems reasonable to suspect differences in metabolic behavior between, on the one hand, the amino nitrogen and the carbon skeleton of glycine, and, on the other hand, between the methylene and the carboxyl carbon atom, our findings on a variety of tissues, and on urine, feces, and expired CO_2, may be of interest. No attempt has been made to account for all of the C^{14}-activity administered.

Methods

Glycine was administered to young adult male rats (ranging from 200 to 275 g. body weight) by one of three routes: (1) intravenously (4 rats), (2) intraperitoneally (1 rat), and (3) by stomach tube (2 rats). All rats received 1 μc. of the C^{14}-labeled amino acid, with the exception of rats No. 13 and No. 21 (*cf.* Table I) which received 2 μc. The rats were allowed free access to a diet of purina fox chow and water until the time of glycine administration, after which all food was withdrawn. All but one of the animals were sacrificed and processed 24 hr. after the administration of glycine, as described previously (1). Rat No. 55 was killed 5 hr. after the injection of glycine.

In all experiments glycine containing C^{14} in its α-carbon atom and having a C^{14}-

[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

activity of 4.0×10^6 disintegrations/min. (= 1.83 μc.)/mg. was used.[2] All isotope measurements were made by the method of Bale and Masters, as briefly described elsewhere (1). After administration of glycine all animals were placed in metabolism cages to permit separate collection of urine and feces. The total amount of urine excreted after glycine injection was diluted to 10 ml. with water and then aliquots were taken for C^{14}-analysis, and for the preparation of dixanthyl urea according to Fosse (8). The tissues and the gastrointestinal tract contents were dried by lyophilizing, whereas fecal samples were dried in air and then over P_2O_5 *in vacuo*. In one experiment, expired CO_2 was collected in NaOH traps and aliquots were then taken for C^{14}-analysis, CO_2 being liberated by acid addition.

Results

Results pertaining to tissue concentrations of C^{14} are presented in Table I, where C^{14}-activity is expressed in terms of "corrected" C^{14}-activity[3] in order to permit comparison of specific activities. The total amount of C^{14} incorporated into the tissues is given as percentage of the total C^{14}-activity administered, based on the total dry weight of the tissue as determined experimentally.

As might be expected from the magnitude of the contribution of muscle to the total body weight, the general mass of dissectable skeletal muscle contains the highest percentage of the total administered activity, although the C^{14}-concentration[4] of this tissue is low. The C^{14}-concentration of diaphragm is higher than that of other skeletal muscle (except in the case of rat No. 55). This observation is probably attributable to the higher physiological activity of diaphragm as compared to other skeletal muscle. Similarly, the heart, another muscle of high physiological activity, also shows a higher C^{14}-concentration than the large mass of skeletal muscle analyzed. The highest C^{14}-concentrations were found in the gastrointestinal tract, liver, kidney, and lungs. Brain was found to have the lowest C^{14}-concentration of all tissues examined. Since preliminary fractionations of brain (rats No. 55, A32, and 28) have indicated that only approximately 30% of the total C^{14}-activity of the brain resides in the protein fraction (freed from

[2] We are indebted to Dr. B. M. Tolbert of the Radiation Laboratory of the University of California for making available this preparation of pure glycine synthesized by Dr. R. Ostwald.

[3] "Corrected" C^{14}-activity: $10^{-3}\,\dfrac{\text{disintegrations/min./g. dry weight of tissue}}{\text{disintegrations/min./kg. body weight}}$.

[4] That is, disintegrations/min./g. dry weight of tissue.

TABLE I

	Rat No. 13		Rat No. 21		Rat No. A32		Rat No. 28		Rat No. 43		Rat No. 110		Rat No. 55	
	Stomach tube		Stomach tube		Intraperitoneal		Intravenous		Intravenous		Intravenous		Intravenous	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Brain	0.16	0.05	0.40	0.17	0.99	0.12	0.96	0.09	1.17	0.14	0.63	0.09	1.02	0.12
Muscle—found	1.58	8.89	1.04	7.91	1.95	11.43	2.36	16.36	2.52	18.33	2.03	13.10	1.72	10.70
Muscle—calculated[a]						25.00		30.35		30.45		27.70		21.20
Testes	6.98	1.10	1.30	0.22	4.25	0.62	2.63	0.30	2.67	0.36	2.16	0.42	2.43	0.27
Heart	2.74	0.20	1.63	0.14	2.88	0.15	3.44	0.23	3.44	0.18	2.92	0.22	2.65	0.17
Spleen	6.07	0.26	4.74	0.27	5.11	1.12	5.92	0.78	4.05	0.27	5.87	0.45	5.07	0.23
Kidney	9.89	1.85	2.59	0.53	7.65	1.33	7.11	1.35	5.11	0.81	6.13	1.48	8.35	1.58
Liver	12.72	11.55	2.41	2.18	6.07	6.27	7.34	5.87	9.46	7.22	5.00	5.63	7.28	8.00
Gastrointestinal tract[b]	4.06	3.80			9.38	8.57	7.87	6.22	9.90	7.27	8.52	9.07	6.84	6.63
Pancreas					3.67	0.43	5.63	0.32	3.29	0.36			10.93	2.20
Diaphragm					4.10	0.15	3.06	0.12					1.54	0.08
Duodenal mucosa[c]													10.21	1.81
Lungs													8.55	0.82
Bone marrow[d]					5.50		7.80		6.80		4.30		19.65	
Gastrointestinal contents[e]		0.82						1.16		0.86		2.25		
Feces		3.58		2.48		0.91		0.91		0.64		2.23		
Urine		9.95		6.45		0.50		7.36		0.54		5.85		

1—Corrected C^{14}-activity.
2—Per cent of total administered C^{14}-activity.

[a] The calculated value for the total percentage of administered activity present in muscle is derived from the theoretical total dry weight of muscle as related to the total body weight (9).

[b] After dissection, the gastrointestinal tract was washed out carefully with saline before lyophilizing.

[c] In this case, the duodenum was separated from the remaining gastrointestinal tract. The duodenal mucosa was then removed by gentle pressure on the duodenum.

[d] Bone marrow was removed from the femora and analyzed in the wet state. The figures reported have been calculated on the assumption that bone marrow contains 20% solid materials.

[e] The gastrointestinal contents were removed from the intestinal tract by washing with saline. The manipulations which are involved in obtaining the total dry weight of the gastrointestinal contents are estimated to contribute an error of approximately 10%.

nucleic acid)[5], it is at present difficult to state whether the slow rate of turnover of brain protein is the cause of its low C^{14}-concentration. In two experiments (rats No. 28 and 55) the liver was fractionated and approximately 85–90% of the total activity was found to be present in the protein fraction (from which nucleic acids had been removed).

The elimination of the α-carbon atom of glycine as CO_2 within a period of 5 hr. after intravenous injection (rat No. 55) accounts for 11.8% of the total activity administered. It appears that the methylene carbon atom of glycine differs in this respect from the carboxyl carbon atom, since the rate of elimination of the carboxyl carbon atom labeled with C^{14} (in mice) is very rapid, approximating that of the carboxyl group of acetate (6). The data presented below also indicate that the largest percentage of the C^{14}-activity expired as CO_2 is eliminated in the first hour. The CO_2 collections were made within the 5-hr. experimental period and analyzed for C^{14} with the following results:

	Total C^{14}-activity administered
	per cent
First hour	4.91
Second and third hour	5.71
Fourth and fifth hour	1.20

The urinary and fecal excretion of C^{14}-activity are also shown in Table I. As can be seen, the amount of C^{14}-activity excreted through these pathways is relatively small, ranging up to 10%. In two cases (rats No. 21 and 13) urea was isolated from 1 ml. of diluted urine (as dixanthyl urea) and was found to have C^{14}-activities of 1.19 and 0.99 (10^4 disintegrations/min./mM urea), *i.e.*, 1.7% and 2.3% of the total urinary activity or 0.15% and 0.17% of the total C^{14}-activity administered.

Comments

As can be seen from Table I, animals which were given labeled glycine by intravenous and intraperitoneal routes show reasonably good agreement (allowing for a 10% experimental error) with respect to tissue isotope concentration, while animals given glycine by stomach tube exhibited wider variations. Since the animals fed by stomach tube were not fasted prior to the administration of the amino acid, such

[5] These fractionations of brain also revealed the presence of approximately 60-65% of the total C^{14}-activity of the brain in the lipide fraction. The lipides of the gastrointestinal tract were also found to contain significant C^{14}-activity in several cases.

variations may be due to differences in the rate of amino acid absorption. It is also of interest to note that there appears to be relatively little difference between the 24-hr. rats and the 5-hr. animal. The only marked difference appears to be in the case of muscle, whose C^{14}-concentration is lower in the 5-hr. rat, and in the case of the pancreas which shows a considerably higher C^{14}-concentration in this animal.

The data indicate that C^{14}-incorporation into the gastrointestinal tract in animals which were given labeled glycine by intravenous and intraperitoneal routes was slightly greater than into the liver, whereas incorporation into the kidney was of approximately the same order as liver in this group of animals. Skeletal muscle, in general, seems to contain about 1/3 of the activity of liver, whereas the ratio of diaphragm or heart to liver C^{14}-activity is somewhat higher. Because of the differences in route of administration and labeling, the relationships between the C^{14}-activity of the liver, internal organs and muscle differ from those found by other workers (3).

Sprinson (10) reported data on the N^{15}-excretion in man fed N^{15}-labeled glycine showing that 12% of total N^{15}-activity was excreted within 5 hr., and 25–30% within 24 hr. These values are somewhat higher than the values reported here. Greenberg and Winnick's (5) value of 3.7% total activity excreted in urine is not comparable to our values since the collection period is not identical.

Although quantitative data presently available are limited, it seems likely that a large portion of the C^{14}-activity of the tissues is actually a measure of protein metabolism, except in the case of brain and gastrointestinal tract, which has been discussed before. The differences in the anabolic and catabolic activity, particularly as concerns proteins, are reflected in the wide variations in C^{14}-activity in the individual tissues.

Acknowledgments

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Summary

1. Data on the distribution in a variety of rat tissues of the methylene carbon atom of glycine labeled with C^{14} have been presented, in terms of corrected C^{14}-activity and percent of total administered C^{14}-activity.

2. Urinary and fecal excretion of C^{14} subsequent to administration

of labeled glycine, as well as C^{14}-activity of the gastrointestinal contents have been reported. In two cases, data on C^{14}-activity of urea have been presented.

3. The C^{14}-activity of CO_2 expired over a period of 5 hr. was measured and found to constitute *ca.* 12% of the total C^{14}-activity administered.

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Esterified Fatty Acid Levels of Normal Human Sera

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Introduction

Recently the authors described a simple colorimetric procedure for determining total esterified serum fatty acids (1). The method is based upon the conversion of the fatty acid esters into the corresponding hydroxamic acids by hydroxylamine hydrochloride, and their subsequent conversion to colored ferric salts. The present report records the esterified fatty acid content of fasting sera from 102 apparently normal persons, and compares these with values published by various authors (Table II).

Materials and Methods

Laboratory technicians, nurses and other hospital employees reported to the laboratory without breakfast at about 8:00 A.M. Blood was drawn and the serum esterified fatty acid content was determined in triplicate or duplicate according to the method described (1). Most of the subjects were young women.

Results

The results of the analyses are contained in Table I. The maximum is 12.6, the minimum 7.0, and the mean is 9.2 meq. of esterified fatty acids/l. of serum. The standard deviation is $\pm$ 1.29.

Comments

Since free fatty acids in sera are not measured by this procedure, the results may not be exactly comparable to values obtained in procedures

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measuring total fatty acids. However, the free fatty acid contents of human sera have been reported to be small (2), calculated at 3% or less of the total fatty acid content (3).

Plasma or serum total fatty acids have been determined by procedures employing various principles. Bloor (4) and Boyd (5) determined total fatty acids and cholesterol on alcohol-ether extracts of plasma by saponification, extraction of the acidified residue with petroleum ether, and oxidation of an aliquot of the solution with H_2SO_4–$K_2Cr_2O_7$ reagent. Cholesterol was determined separately in another aliquot, and its oxidation value calculated and subtracted from the oxidation value of the mixture of total lipide, giving the value for total fatty acid.

TABLE I

Serum Esterified Fatty Acid Content of 102 Normal Fasting Individuals Grouped According to Levels

Esterified serum fatty acid levels meq./l.	No. of persons
7.0–7.9	18
8.0–8.9	27
9.0–9.9	31
10.0–10.9	13
11.0–11.9	7
12.0–12.9	6

Stoddard and Drury (6), Man and Gildea (7), and Thannhauser (12), extracted the serum or plasma with alcohol and ether, then saponified the extract. The soaps were suspended or dissolved in water, and HCl was added to cause separation of the free fatty acids. The latter were recovered by filtration through a Gooch filter. Finally, the fatty acids were redissolved in hot alcohol and titrated with standard alkali. Wilson and Hansen (8) saponified alcohol-ether extracts of sera, evaporated these to dryness, added water, acidified, and extracted with petroleum ether. The fatty acids were removed from the petroleum ether with alcoholic KOH solution and water, and were recovered by evaporating this to dryness, dissolving in water, precipitating with acid and extracting with petroleum ether. The fatty acids were then weighed and titrated.

Table II gives the serum fatty acid values obtained by the various authors.

When the minimum values of total serum fatty acid levels in Table II are compared, all are at approximately the same level (6.9–8.6

meq./l.). The range of fatty acid levels in many of the different series is large, with the exception of our series, wherein the range is remarkably small. The reason for the small range observed by this method of analysis, in contrast to the broad range in other series, is not clear. It seems probable that the specificity of the colorimetric reaction involved may be partly responsible, for only fatty acids present in ester form react. Other organic compounds, such as urea and amino acids, do not produce a color under the conditions of analysis.

TABLE II

Normal Serum or Plasma Fatty Acid Levels Obtained by Various Authors

Authors	No. of sera analyzed	Meq. of fatty acids/l.			
		Maximum	Minimum	Mean	S. D.
Peters and Man (9)	355	36.9	7.3	12.3	±3.37
Boyd (5)[b]	8	16.1	8.6	12.5	±1.77[a]
Bullen and Bloor (10)[b]	12	19.9	8.3	11.8	±3.44[a]
Wilson and Hansen (8)[d]	9	18.2	7.5	13.0	±2.92[a]
Thannhauser (12)[c]	—	16.2	7.2	—	—
Stoddard and Drury (6)[c]	10	12.0	6.9	10.6	—

[a] S. D. was calculated from the authors' data after converting their values to meq./l., where necessary. The following formula was used:

$$\text{Standard deviation} = \sqrt{\frac{\text{Sum of squares of deviations from mean}}{\text{Total number of observations}}}.$$

[b] Meq. were calcualted from fatty acid weights (mg.-%) by the following formula: $\frac{\text{Fat(mg.-\%)}}{28.3} = \text{meq. fatty acid/l.}$ (11).

[c] In the method of Stoddard and Drury (6) the average fatty acid molecular weight is assumed to be 277.2 when fatty acid weight is calculated from their titration values. Therefore, the following formula is used to convert data obtained by their method back to meq./l.:

$$\frac{\text{Fat(mg.-\%)}}{27.72} = \text{meq. fatty acid/l.}$$

[d] Data from serum of individuals with illnesses is excluded.

Other factors, in at least one study (10), may be time when blood was drawn for analysis, *i.e.*, about 3 hr. after a meal; whereas in our series, blood was drawn after an all-night fast (about 12 hr.) and after the subjects had come for work to the hospital. The effect of slight muscular activity on serum lipides is not completely understood (11).

Summary

Serum esterified fatty acids were determined on 102 healthy fasting individuals. The maximum was 12.6, the minimum 7.0 and the mean 9.2 meq./l. A brief comparison is made between the fatty acid levels found in this study and similar data obtained by other authors.

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Factors Influencing Oxygen Production by Illuminated Chloroplast Fragments

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Introduction

One of the serious obstacles in the experimental study of the mechanism of photosynthesis has been the impossibility of separating the process from the activities of intact green cells. In recent years, however, an important advance was made by R. Hill (1,2), who demonstrated that the long-known capacity of isolated chloroplasts to evolve oxygen can be greatly enhanced by the use of suitable oxidants.

Although the entire process of photosynthesis was not reconstructed in the chloroplasts, in the sense that in no case was CO_2 reduction linked with oxygen evolution (3), yet the work of Hill made it possible to investigate outside the living cell the reaction most characteristic of photosynthesis in green plants: the photolysis of water resulting in the evolution of gaseous oxygen. The recent investigations in this field have been reviewed by Holt and French (4), who marshaled the evidence in favor of the identity of the oxygen-liberating mechanism in isolated chloroplasts with that in the intact green cells.

Despite the substantial measure of agreement among the investigators of isolated chloroplasts, there remained several areas of conflicting observations, some of them of considerable theoretical importance. The present communication deals with a part of our investigation of the reactions of isolated chloroplasts, in which a special attempt was made to examine the points of discordance.

Methods

Chloroplast fragments, rather than whole plastids, were used in all experiments. The preference for fragments was in accord with the general objective of using the smallest subcellular, chlorophyll-bearing aggregate still capable of evolving oxygen upon illumination. The chloroplast fragments were prepared from large leaves of chard

(spinach beet), *Beta vulgaris*, grown in a greenhouse in a nutrient solution of the composition previously used in sand culture (5). The procedure for preparing chloroplast fragments was the same as previously described (5), except that operations were carried out in the cold and the leaves were shredded with a stainless steel knife, prior to grinding for 2 min. in a Waring Blendor in the presence of $M/15$ potassium phosphate buffer of pH 6.5.

Chlorophyll was determined (5) for each batch of chloroplast fragments and an aliquot selected to give approximately 0.5 mg. of chlorophyll ($a + b$) in each manometer vessel. With quinone and ferricyanide, the quantity of oxidant used was also standardized to yield 84 mm.^{3} of O_2 for each vessel. Standard reagents without further attempt of purification were used. Solutions of three oxidants were prepared, just prior to use. Forty mg. of quinone were dissolved in 10 ml. 0.01 N H_2SO_4. The use of acid as a solvent was based on the recommendation of Warburg and Lüttgens (6). With the quinone at our disposal, water solutions were equally satisfactory if used without delay. No difference was observed between samples of quinone from two different sources: Eastman-Kodak and Hopkins and Williams (England). Unless otherwise indicated, 0.2 ml. of the acid quinone solution containing 7.5×10^{-6} moles was added to each vessel. The ferricyanide was prepared by dissolving 2.0 g. of $K_3Fe(CN)_6$ in 100 ml. $M/15$ phosphate buffer, pH 6.5. Twenty-five hundredths ml. of the solution, corresponding to 15×10^{-6} moles, was added to each vessel. Of the phenol indophenol (approximately 41% pure as determined by ascorbic acid titration) 30 mg. were dissolved in 5 ml. phosphate buffer, pH 6.5, and 0.5 ml., corresponding to approximately 5.1×10^{-6} moles, were added to each vessel.

Oxygen evolution was measured manometrically at 15°C. in a refrigerated constant temperature bath of a design similar to that developed by S. Aronoff, equipped with a plate glass strip at the bottom. Illumination was provided by a battery of 150 watt Mazda projector flood lamps placed under the glass strip. The intensity of illumination at flask level was approximately 28,000 lux. At this relatively high level of illumination the rate of oxygen evolution was essentially independent of light intensity. All readings were made in an atmosphere of nitrogen. No attempt was made to purify the nitrogen gas from traces of oxygen. The total volume of the reaction mixture was 3 ml., and all measurements were made in conical vessels of approximately 15 ml. capacity.

Yields and Rates

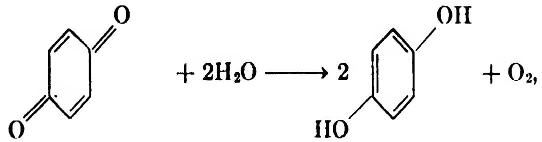
The overall equation of photosynthesis,

$$\text{(i)}\quad CO_2 + 2\,H_2\overset{*}{O} \longrightarrow \{CH_2O\} + H_2O + \overset{*}{O}_2,$$

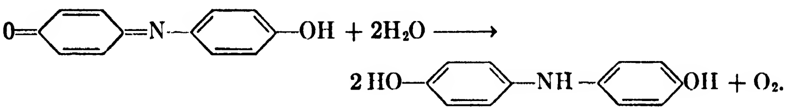
indicates that the release of each oxygen atom from the splitting of water is accompanied by the release of two hydrogen atoms (7). If the oxygen-liberating mechanism in photolysis is the same as in photosynthesis,[1] an identical relation between hydrogen and oxygen must

[1] In discussing the evolution of oxygen from the splitting of water by illuminated chloroplasts, it is convenient to adopt a short-hand expression analogous in usage to such terms as photosynthesis, photooxidation and photoreduction. The term photolysis has been adopted for this purpose.

obtain. It becomes important, therefore, to consider whether stoichiometric yields of oxygen are obtained in photolysis reactions with several oxidants in accordance with the following equations for quinone, ferricyanide and phenol indophenol respectively:

(ii) 2 

(iii) $4\ K_3Fe\ (CN)_6 + 4\ K^+ + 2H_2O \longrightarrow 4\ K_4Fe\ (CN)_6 + 4H^+ + O_2,$

(iv) 2 

Warburg and Lüttgens (6), who discovered the quinone reaction, reported yields between 80 and 90% of theoretical. On the other hand, Aronoff (8) was unable to obtain with quinone oxygen yields higher than 35% of those expected from Eq. (ii). Aronoff (8) attributed his low yields to competing reactions resulting from the deterioration of quinone. Warburg and Lüttgens (6) explained their slight discrepancy from theoretical yields by the utilization of oxygen by chloroplast fragments, concomitant with the much greater photolytic oxygen evolution.

Warburg and Lüttgens (6) obtained their high yields only upon the addition to the chloroplast fragments ("granula") of an accessory factor contained in the supernatant fluid remaining after centrifuging off the suspension containing the chlorophyll. They identified this accessory factor as chloride and designated this anion as the "coenzyme" of photolysis.

With quinone as the oxidant, we obtained yields of oxygen at 15°C. (Table I) in accord with theory (Eq. ii). The marked effect of chloride reported by Warburg and Lüttgens (6) was confirmed in this and numerous other experiments. The absence of this ion may account for the low yields obtained by Aronoff (8). Striking as is the effect of chloride on oxygen evolution, other evidence at hand does not support the conclusion of Warburg and Lüttgens (6) that chloride is a coenzyme for oxygen evolution. A full discussion of this subject is reserved for another paper. For the purpose of the current investigation, the addition of chloride to the reaction mixture was adopted as a standard

TABLE I

Oxygen Evolution by Chloroplast Fragments

Oxidant	Yield (theory) *per cent*		Rate ($Q_{O_2}^{chl}$)[a]	
	No KCl	0.01 *M* KCl	No KCl	0.01 *M* KCl
Quinone	36	102	290	1030
Ferricyanide	19	102	200	800
Phenol indophenol	90	107	400	730

[a] $Q_{O_2}^{chl}$ = mm.³ of oxygen/hr./mg. of chlorophyll, computed from data obtained for the 6-min. period from 1 min. to 7 min. after turning on the light.

procedure to insure maximum yields and rates as points of reference necessary for evaluating the influence of various factors on photolysis.

As already noted by Warburg and Lüttgens (6), the activating effect of chloride was not confined to quinone, but was also apparent when ferricyanide was used as an oxidant (Table I). In agreement with these authors, and with Holt and French (11), we have also found that $K_3Fe(CN)_6$ alone can act as the oxidant in photolysis without the addition of the other reagents in the original reaction mixture as used by Hill and Scarisbrick (9) or in the modified formula used by Holt and French (10). In our experiments, supplementing the ferricyanide with $K_2C_2O_4$, or with ferric iron in the presence of oxalate, produced no increase in the yield or rate of oxygen evolution. The illumination of reaction mixtures containing appreciable amounts of ferric oxalate was found under our conditions to yield large amounts of CO_2. Thus, in the absence of KOH, the addition of ferric oxalate may give apparent high yields owing to the inclusion of evolved CO_2 with the oxygen measured.

The suitability of phenol indophenol as an oxidant for quantitative mesaurement of oxygen evolution by chloroplasts was demonstrated by Holt and French (11). Our own results with this dye are given in Table I. As with the other two oxidants, stoichiometric yields of oxygen were obtained with this dye as well. There was a marked chloride activation on the *rate* of oxygen evolution, but it was less in magnitude than with the other oxidants. It is possible that the impurities in the dye included a small amount of chloride, not sufficient, however, to bring about maximum rates. Unlike the other oxidants, phenol indophenol gave high *yields* even in the absence of added chloride. It is believed that this is attributable to the relatively high $Q_{O_2}^{chl}$ (Table I) obtained without chloride. A discussion of the relation between rates and yields in a later section of this paper has a further bearing on this point.

Effect of the Cytoplasmic Fluid

The high-speed centrifugation used for separating the chloroplast fragments yielded a pale yellowish-green supernatant, which contained only a minute amount of chlorophyll. It was designated, as previously

(5), the cytoplasmic fluid. For example, in a typical preparation the suspension of chloroplast fragments contained 1.9 mg., as compared with 0.01 mg. of chlorophyll/ml. in the cytoplasmic fluid. As far as preparatory technique is concerned, our chloroplast fragments corresponded to the "granula" of Warburg and Lüttgens (6) and the "grana" of Aronoff (8), whereas our cytoplasmic fluid corresponded to the "solution" of Aronoff (8).

Warburg and Lüttgens (6) found no evolution of oxygen upon illumination of the cytoplasmic fluid alone. On the other hand, Aronoff (8) reported a high rate of oxygen evolution in the illuminated cytoplasmic fluid ("solution"), about 10 times as great as that of the chloroplast fragments. The chlorophyll concentration in Aronoff's cytoplasmic fluid was not given, but it was assumed that it was low as in our preparations. A question of fundamental interest was thus raised: is there some constituent of the cytoplasmic fluid, other than the previously noted chloride, capable of enhancing the rate of photolysis by chloroplast fragments?

To test this possibility, the following experiments were carried out. A reaction mixture was prepared with quinone as the oxidant, containing a small amount of chloroplast fragments computed to give the same concentrations of chlorophyll as in the cytoplasmic fluid. The results of one experiment, summarized in Table II, disclosed that, when minute amounts of chlorophyll were used, the cytoplasmic constituents failed to increase the rate of oxygen evolution. The actual oxygen evolution in the vessel containing the small amount of chlorophyll was very much lower than that in the vessel with the high (0.5 mg.) chlorophyll content: 4–6 *vs.* 45 mm.³ of oxygen (Table II). Upon inserting the respective figures into the formula, $Q_{O_2}^{chl} = \frac{\text{mm.}^3 \text{ } O_2 \text{ evolved in 6 min.}}{\text{mg. chlorophyll}} \times 10$, higher rates were obtained for the

TABLE II

Oxygen Evolution from Cytoplasmic Fluid and from Chloroplast Fragments, Containing Varying Amounts of Chlorophyll

	Chlorophyll concentration	mm.³ of O_2 evolved in 6 mins.	$Q_{O_2}^{chl}$
Cytoplasmic fluid	0.032	4	1250
Chloroplast fragments	0.032	6	1870
Chloroplast fragments	0.49	45	920

two reaction mixtures containing the low chlorophyll concentration (Table II). It is doubtful, however, whether the differences are significant. An error of 1 or 2 mm.[3] of oxygen would be multiplied many times and have a marked effect on the $Q_{O_2}^{chl}$ for 0.032 mg. of chlorophyll, but it would only slightly change the computed rate for 0.5 mg. chlorophyll. It is for this reason also that we ascribe no special significance to the difference in the rates between the two reaction mixtures containing the low concentrations of chlorophyll.

Stability of the Photolytic System

In Intact Leaves

Warburg and Lüttgens (6) found no loss in activity of chloroplast fragments after storing sugar beet leaves for several days at 5°C. Under our conditions, a comparison (with quinone as the oxidant) of the activity of chloroplast fragments of freshly picked spinach beet leaves with that of leaves kept for 4 days in the dark at 2°C. in a pliofilm bag to insure turgidity showed a loss on storage of about 25%: $Q_{O_2}^{chl}$ of 1010 and 740, respectively.

The leaves used in the present investigation were generally harvested in the morning but, on a number of occasions, were used after dark storage overnight without any apparent loss of photolytic activity of the chloroplast fragments. Hill and Scarisbrick (13), working with *Setellaria media*, stressed the importance of time of day for collecting leaves: highest activity of chloroplasts was obtained in leaves picked in the morning, the activity falling nearly to zero in leaves picked during a sunny afternoon. Kumm and French (12) observed an increase in activity following illumination of leaves previously stored in the dark, just prior to the separation of chloroplasts. Under our conditions, no such dependence of activity on light or time of day was observed. In one experiment, leaves were collected in the greenhouse at about midnight and kept in the dark at 2°C. until the chloroplast fragments were separated the following afternoon. All manipulations, prior to the turning on of light for the photolysis reaction, were carried out in dim light. The $Q_{O_2}^{chl}$ obtained with quinone as the oxidant was 1010, comparable to that of active preparations obtained from leaves harvested at other times of the day.

Chloroplasts and Chloroplast Fragments

The experiments of Hill and Scarisbrick (13) and Kumm and French (12) with intact chloroplasts indicated a rapid loss of photolytic activity with storage, with half-lives of the order of 2 hr. French, Anson and Holt (14) found, however, much greater stability in chloroplasts stored at 0°C.: between 25 and 50% of the original activity was lost in 12 hr. and, in some preparations, even smaller losses occurred in that period.

There is agreement among different investigators that the photolytic mechanism in the chloroplast fragments is relatively more stable. Warburg and Lüttgens (6) found that chloroplast fragments when stored in $M/20$ phosphate buffer at pH 6.21, containing 0.5% KCl, lost only 5–15% of their activity in 24 hr. The half-life of a similar preparation stored at 2–3°C., presumably without KCl, by Aronoff (8) was 11 hr. Our own preparations of chloroplast fragments stored in phosphate buffer at 2°C. were also relatively stable, the loss of activity in 5 hr. amounting to about 14% ($Q_{O_2}^{chl}$ of 1190 *vs.* 1020).

A striking rate of deterioration of photolytic activity was observed, however, in chloroplast fragments prepared by another procedure. Instead of grinding in a Waring Blendor in phosphate buffer, batches of leaves were macerated in the cold in a "Vitajuicer"[2]. This instrument permitted a trituration of leaves without the use of liquid, accompanied by rapid separation of the green leaf juice undiluted by an extraneous solvent. The dark green fluid was then handled in the same manner as the previously described filtered Blendor mash (5): *i.e.*, centrifuged at low speed for 1 min., the residue discarded and the low-speed centrifugate (l.s.c.) centrifuged for 20 min. at high speed to separate the chloroplast fragments from the cytoplasmic fluid.

The stability of the photolytic system in the chloroplast fragments divested of the cytoplasmic fluid was as in the Waring Blendor preparation, but a rapid deterioration was noted in the activity of the l.s.c., that is, when the chloroplast fragments were stored (at 2°C.) in combination with the cytoplasmic fluid:

Hours after grinding leaves	0.75	2.00	4.3	7.75
$Q_{O_2}^{chl}$	1600	1200	740	490

[2] Distributed by Enterprise Development Corp., 231 W. Olive Ave., Burbank, Calif.

The very high $Q_{O_2}^{chl} = 1600$, obtained within 45 min. of grinding the leaves, and the rapid falling off of activity thereafter, gives a measure of the rate of inactivation occasioned by the presence of the cytoplasmic fluid. It also suggests that an unavoidably large degree of deterioration takes place during the relatively long period of high-speed centrifugation when the cytoplasmic fluid and the chloroplast fragments are combined.

Inactivation by Heating

Isolated chloroplast fragments when held for 5 min. at 55°C. failed completely to evolve oxygen even with the addition of KCl (using quinone as the oxidant). The sensitivity of the photolytic system to heat was previously shown by Warburg and Lüttgens (6), who found complete inactivation by heating for 10 min. at 50°C. Holt and French (10) have also shown that maintaining chloroplasts at 35°C. for 15 min. resulted in the loss of 80% of the photolytic activity (with ferricyanide as oxidant), while 5 min. at 35°C. caused a loss of 37%.

The thermolability of the photolytic system was found to be in distinct contrast with the thermostability of the polyphenoloxidase known to occur in the chloroplast fragments (5). The polyphenoloxidase activity remained virtually unchanged after heating the material at 75°C. for 5 min. The oxidase activity was lost after heating at 100°C. for 3 min.

Sensitivity to Light

Warburg and Lüttgens (6) reported that illumination of suspension of chloroplast fragments in the absence of an oxidant rapidly destroyed the photolytic activity when subsequently the oxidant was added. Since it was not clear whether this inactivation was peculiar to the quinone which these authors used, nor whether the pre-illumination included the oxidant as well as the chloroplasts, we undertook to re-examine this point with the use of 3 oxidants: quinone, ferricyanide and phenol indophenol.

Chloroplast fragments were placed in the main vessel and the respective oxidants in the side-arm. The main vessel and the side-arm were appropriately darkened with tinfoil or exposed to light prior to mixing in accordance with the outline given in Table III. The "dark exposure" of either the chlorophyll fragments or the oxidant served as a check on the thermal deterioration during the 20 min. of exposure to light—an important point in view of the thermolability of the chloroplast material.

The pretreatment, either in light or dark, was given while the vessels were undergoing shaking in a bath kept at 15°C. In the control treatments aliquots of the same suspension of chloroplast fragments were mixed with the respective oxidant at zero time.

The results presented in Table III indicate that the thermolability of the shaken chloroplast fragments during a 20 min. period can account for serious losses in activity. Moreover, the three oxidants varied in their respective photo- and thermosensitivity. Quinone was very unstable in light and an exposure of 20 min. rendered it unsuitable as an oxidant for the chloroplast fragments. An indication of the instability of phenol indophenol was also obtained. The ferricyanide was found to be stable either in light or in dark and it was thus most suited for the

TABLE III

Effect of Preillumination of Chloroplast Fragments (chl.f.) and Oxidants on Oxygen Evolution

Pretreatment was given in vessels undergoing shaking in a bath kept at 15°C.

Experimental conditions	$Q_{O_2}^{chl}$		
	Quinone	FeCy	Phenol indophenol
1. Chl.f. and oxidant mixed at 0 time	1080	800	700
2. Chl.f. preilluminated, oxidant kept in dark. Mixed at 20 min.	530	560	300
3. Chl.f. kept in dark, oxidant preilluminated. Mixed at 20 min.	140	600	260
4. Chl.f. and oxidant kept in dark. Mixed at 20 min.	610	560	300

evaluation of changes in the chloroplast preparation. When due allowances are made for the thermal inactivation of the chloroplast fragments and the peculiarities of the oxidants, the data in Table III offer no basis for a conclusion of light inactivation in the absence of oxidant. It will be recalled that these experiments were carried out in an atmosphere of nitrogen. Data obtained by one of us (Arnon) in another investigation showed that, in air, the illumination of chloroplast fragments in the absence of an oxidant resulted in a marked photooxidation.

Effect of Temperature on Rates and Yields

The thermal instability of the photolytic system in chloroplasts is further illustrated by the data in Table IV. The highest rates of oxygen evolution were obtained at the lower temperatures. This is in accord with Holt and French (10) who observed highest activities at about 10°C. It seems probable that the decreased activity at higher temperatures is a reflection of an accelerated destruction of enzymes or some unstable intermediates essential to the light reaction.

TABLE IV

Effect of Temperature on Rate of Oxygen Evolution with Different Oxidants

	$Q_{O_2}^{chl}$			
	10°C.	15°C.	20°C.	25°C.
Quinone	700	920	820	660
Ferricyanide	620	700	740	460
Phenol indophenol	520	730	720	620

An interesting relation between temperatures and total yields of oxygen is illustrated in Table V. As previously noted (Table I) the omission of KCl was associated with a low rate of oxygen evolution. The corollary of a lower rate is an increase in the time required for the oxygen evolution to come to completion, *i.e.*, to reach the amount required by the stoichiometric relations (100% yield). But this delay occasioned by a slower rate is conducive to the deterioration of the unstable photolytic system, especially at the higher temperatures.

TABLE V

Effect of Temperature on the Yield of Oxygen

	Percentage yields							
	No KCl				KCl Added			
	10°C.	15°C.	20°C.	25°C.	10°C.	15°C.	20°C.	25°C.
Quinone	70	51	38	19	101	101	107	94
Ferricyanide	43	32	27	17	100	88	88	76

Thus, as shown in Table V, the per cent yield falls off precipitously with higher temperature in the "minus KCl" series, whereas only a mild decline was observed in the "plus KCl" preparations. Even within the "plus KCl" group, the lower rates of the ferricyanide series (Table IV) are also reflected in a greater decline in yields. The attainment of a theoretical yield of oxygen seems to be dependent on the photolytic mechanism winning the "race" against time with the concomitant inactivation of the system. The higher the rate, the better are the chances for winning the race. If this interpretation is correct, it holds important implications for studying effects of inhibitors, pH, *etc.*, on the photolytic process. Conclusions about the effects of these factors will have validity only if the control measurements indicate high rates and yields.

Effect of pH

Divergent results were reported on the influence of hydrogen ion concentration on oxygen evolution. Hill and Scarisbrick (13) found an optimum at pH 8, with the activity falling to half at pH 6.5 and vanishing at pH 5.6. Warburg and Lüttgens (6) imply that pH 6.5 was their optimum, the oxygen evolution becoming greatly diminished or stopping altogether at pH 7.4. Holt and French (10) found that the optimum pH varied according to the temperature and method used for measuring the activity of chloroplasts. With ferricyanide at 15°C. the optimum for the evolution of oxygen was pH 7, but with the hydrogen ion measurement technique different optimal values were obtained at different temperatures: pH 7 at 10°C. and pH 7.6 at 3°C.

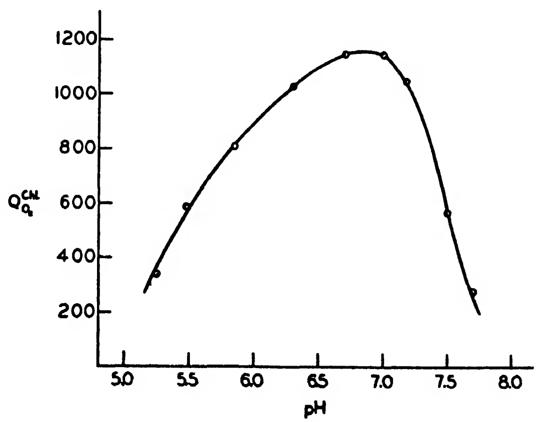


Fig. 1. Effect of pH on oxygen evolution by chloroplast fragments.

The influence of pH from 5.2 to 7.7 under our conditions (0.5 mg. of chlorophyll per vessel, 15°C., quinone) is shown in Fig. 1. All measurements were made in $M/15$ phosphate buffers adjusted to the respective pH values. The final pH at the end of the manometric measurements was determined with a glass electrode. The optimum pH was found to be approximately 6.9. The activity of the system fell off rather sharply on the alkaline side as compared with a more gradual decline on the acid side. It is possible that here again direct observations are complicated by the interaction of two factors: increased activity and accelerated deterioration, both occuring at a slightly alkaline pH. This interpretation, if correct, would account for the apparently more alkaline pH optimum observed at low temperatures by Holt and French (10).

Effect of Inhibitors and Narcotics

Hydroxylamine

This inhibitor is of special significance to the oxygen evolution by chloroplasts and chloroplast fragments. The inhibitory action by hydroxylamine on photosynthesis in intact green cells is attributed to its being a specific inhibitor for the oxygen evolution reaction of the process (15). Hydroxylamine would thus be expected to be the inhibitor *par excellence* if the photolytic system in chloroplasts were indeed the same as the oxygen-releasing mechanism in the intact green cell. Yet, neither Hill (2), using the oxyhaemoglobin method for measuring oxygen output by chloroplasts, nor Aronoff (8), with the quinone method and chloroplast fragments, found any hydroxylamine inhibition. On the other hand French, Anson and Holt (14) found, with ferricyanide as the oxidant, a 72% inhibition with 10^{-4} M hydroxylamine, and Macdowall (16) using the phenol indophenol method obtained a 50% inhibition with 3.1×10^{-4} M hydroxylamine.

Our own results with hydroxylamine are shown in Fig. 2. A marked inhibition of oxygen evolution was obtained, the 50% value corresponding to a concentration of approximately 2×10^{-4} M. It is possible that the absence of inhibition by hydroxylamine observed by Hill (2) and Aronoff (8) was due, in the first instance, to a reaction of the inhibitor with the haemoglobin used and, in the second, to the low activity of the chloroplast preparations to which no chloride was added.

Sodium Azide

As with hydroxylamine, the evidence that this inhibitor of photosynthesis (15) also inhibits oxygen evolution by chloroplast preparations, was contradictory. Hill (2) and Aronoff (8), who measured oxygen evolution by the oxyhaemoglobin and quinone methods, respectively, found no azide inhibition. French, Anson and Holt (14), however, using the ferricyanide test, obtained a complete inhibition of oxygen evolution with 10^{-3} *M* sodium azide, and Macdowall (16) found with the phenol indophenol technique that a 50% inhibition was given by 8×10^{-2} *M* azide.

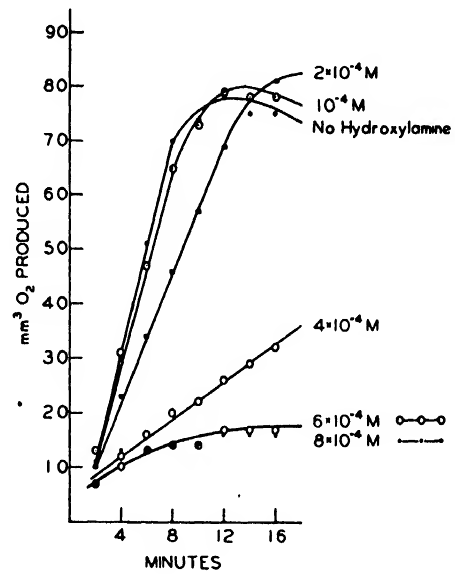


FIG. 2. Hydroxylamine inhibition of oxygen evolution by chloroplast fragments.

The inhibition of oxygen evolution by azide under our conditions is shown in Fig. 3. A 50% inhibition was obtained with a sodium azide concentration of 8×10^{-4} *M*. It is possible that the suggested explanation of the negative results of Hill (2) and Aronoff (8) with hydroxylamine is also applicable to their negative results with azide.

o-Phenanthroline

Warburg and Lüttgens (6) found that the strong metal-binding agent, *o*-phenanthroline, is a powerful inhibitor of the oxygen evolution

by chloroplast preparations. Because of its great effectiveness, a 50% inhibition being given by less than 4.2×10^{-5} *M*, these authors concluded that *o*-phenanthroline is an inhibitor rather than a narcotic. The inhibitory effect of *o*-phenanthroline was confirmed by Aronoff (8), and by Macdowall (16), who found a 50% inhibition with 2.7×10^{-5} *M*.

The striking inhibition of oxygen evolution by phenanthroline was fully confirmed in our experiments, using quinone as the oxidant: a 50% inhibition was obtained with 9×10^{-6} *M* *o*-phenanthroline (Fig. 4). The bearing that *o*-phenanthroline inhibition may have on the participation of a metal in the photolytic mechanism of chloroplasts will be discussed elsewhere.

Phenylurethane

Warburg (17) has shown that phenylurethane acts as a narcotic, inhibiting the light reaction of photosynthesis. A concentration of 5×10^{-4} *M* reduced the photosynthesis of *Chlorella* cells by 50%. Interestingly enough, Warburg and Lüttgens (6) found that phenylurethane produces a similar effect on oxygen evolution by chloroplast fragments: a 50% inhibition was obtained by a concentration of 6.1×10^{-4} *M*. Concordant results were also obtained by Hill and Scarisbrick (13).

Aronoff (8) and Macdowell (16), however, while confirming the narcotic action of phenylurethane, found the effective concentrations to be much higher than those for photosynthesis and photolysis reported above. The former obtained a 50% inhibition with 1.2×10^{-2} *M*, and the latter with 2×10^{-3} *M*. In our experiments the effectiveness of phenylurethane was similar to that found by Warburg and Lüttgens (6) and Hill and Scarisbrick (13). A 50% inhibition was given by 2×10^{-4} *M* phenylurethane.

Summary

1. Illuminated chloroplast fragments yielded stoichiometric quantities of oxygen with 3 oxidants: quinone, ferricyanide and phenol indophenol.

2. Based on chlorophyll content, the rates of oxygen evolution by chloroplast fragments and by the cytoplasmic fluid were comparable.

3. The stability of the oxygen-evolving system under various conditions was investigated.

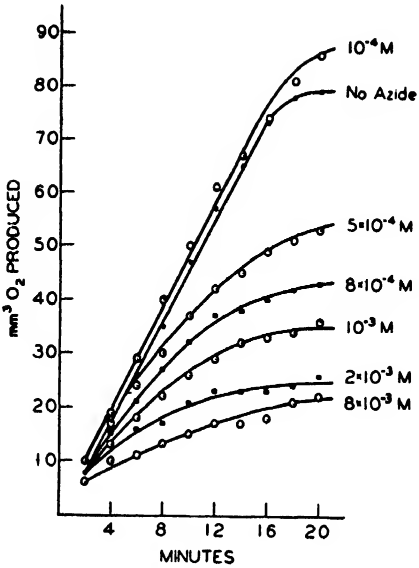


FIG. 3. Sodium azide inhibition of oxygen evolution by chloroplast fragments.

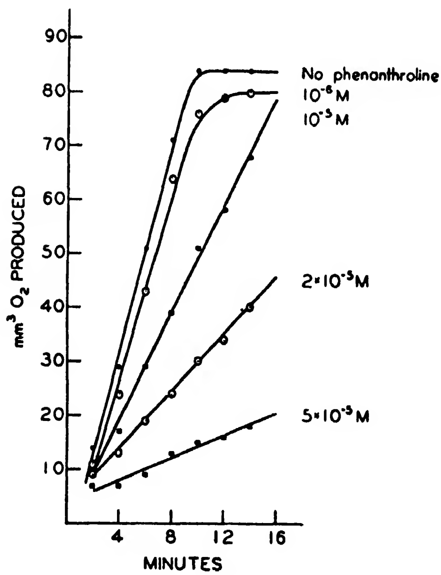


FIG. 4. *o*-Phenanthroline inhibition of oxygen evolution by chloroplast fragments.

4. Support for the identity of the oxygen-liberating mechanism in isolated chloroplast fragments with that in the intact green cells was found in the inhibitory effects of hydroxylamine and phenylurethane. Sodium azide also inhibited the photochemical oxygen liberation by chloroplast fragments. The powerful inhibition by *o*-phenanthroline was confirmed.

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A New Fluorometric Method for the Determination of Epinephrine

When a solution of epinephrine is made strongly alkaline and allowed to oxidize in air, fluorescence may be observed when the exciting wavelength is of the order of 365 mμ (1). This fluorescence is not specific for epinephrine in that we have observed that various substances such as protein hydrolyzates, aged tryptophan, and tyrosine will fluoresce under similar conditions. Further, this fluorescence is transient. The fluorescent material is not completely extractable with organic solvents and the fluorescence is, therefore, affected by salt concentration.

We have been able to convert epinephrine to a substance which will fluoresce when excited by a longer wavelength (*ca.* 435 mμ), which incident light will not excite the interfering substances listed above. This was accomplished by treating the epinephrine solution with ammonia containing an organic primary amine such as ethylenediamine, butylamine, propylenediamine, benzylamine, aniline or *o*-phenylenediamine. A fluorescent product results which is extractable with aliphatic alcohols such as butyl and amyl alcohols. This fluorescence, in contrast to that obtained when epinephrine is treated with alkali, is quite stable and will maintain constant fluorescence for several days. The fluorescence is excited by light of 435 mμ, the secondary filter, having a maximum transmission at 570 mμ, having negligible transmission below 500 mμ. The fluorescence of this material resembles the fluorescence of riboflavin. The reaction probably involves an oxidation of the catechol structure to the quinone and subsequent condensation with the amine. Catechol itself gives a similar reaction.

For the amines containing an aromatic nucleus, such as benzylamine, an excitation wavelength shorter than 435 mμ is needed. We therefore limited our study to the aliphatic amines, particularly ethylenediamine to achieve specificity. Thus, by using a longer wavelength for excitation, and extracting with solvents, we avoid interference from many substances and avoid salt effects.

Distinction from riboflavin itself is made by the ease with which epinephrine may be completely destroyed by oxidizing agents ($KMnO_4$) to which riboflavin is resistant.

In a typical procedure, to 0.05 γ of epinephrine in 1 ml. of 1 N HCl is added 0.5 ml of a 2:1 mixture of 6 N NH_4OH and redistilled ethylenediamine. The mixture is heated on a water bath for 1 hr. at boiling temperature and 5 ml. of redistilled n-amyl alcohol is added, while the mix is still warm. The mixture is vigorously shaken in a test tube with ground glass stopper in which the reaction was carried out. The solution is allowed to cool to room temperature and centrifuged at 2500 r.p.m. The fluorescence of the alcohol layer is measured in a Farrand or Pfaltz and Bauer fluorometer against a fluorescein standard, subtracting a blank due to scattered light, which was run through the same procedure but containing no epinephrine.

The study of the nature of this reaction and its application to the assay of epinephrine in normal and pathological sera is being investigated and will be reported in a more detailed report later.

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The Production of Usnic, Didymic, and Rhodocladonic Acids by the Fungal Component of the Lichen *Cladonia cristatella*

The occurrence of certain organic substances which are peculiar to lichens, has been demonstrated by Zopf (1) and others. The diagnostic value of these substances, which in great part are organic acids, has become a matter of primary importance in the recognition of lichen species as demonstrated by Asahina (2), Evans (3), and others. Lichens and lichen substances, moreover, have recently attracted attention because of their antibiotic properties. In 1944, Burkholder *et al.* (4) demonstrated antibacterial activity of extracts from 27 lichen species against *Staphylococcus aureus* and *Bacillus subtilis* and from 4

against *Alcaligenes fecalis*. In 1945, in a report of further investigations in this field, Burkholder and Evans (5) showed that the primary antibiotic effect against *B. subtilis* was to be attributed to usnic acid, a specific lichen substance. In the same year, Asano *et al.* (6) reported the antibacterial effect of lichesteric acid and its derivatives against staphylococci. Stoll *et al.* (7), in 1947, reported that usnic acid had a high potency of activity against the tuberle bacillus, and Shibata *et al.* (8), in 1948, demonstrated antibacterial action of the same acid against the avian type of this bacillus as well as against *Staph. aureus*.

The fungus and the alga, which constitute a lichen species, have been separated, in certain cases, under aseptic conditions and growth independently in synthetic media, as reported by Tobler (9) and others. Up to the present time, however, very little is known concerning the physiology of either of these organisms when cultured separately. Furthermore, the capacity for the synthesis of the specific substance or substances characteristic of any lichen species has not been established as a function peculiar to the lichen as a whole or to either of its components. In the case of the lichen *Cladonia cristatella* Tuck., however, cultures of the fungal and algal components isolated and grown in synthetic media in this laboratory, when subjected to the microchemical tests developed by Asahina, show that the fungal component is autonomous in the capacity to synthesize usnic and didymic acids. Further evidence in support of this has been supplied by Dr. D. M. Bonner, who demonstrated by paper-partition chromatography the presence of a substance in these cultures that agrees with usnic acid in both R_f value (in phenol saturated with water) and in color reaction with $FeCl_3$. The sample of usnic acid used as a basis for comparison in this demonstration was obtained from Dr. Y. Asahina of the Pharmaceutical Inst., Tokyo Imperial Univ. Rhodocladonic acid, which is normally present in the apothecium of this species, also developed in some of the cultures of the fungal component as indicated by the red pigmentation of portions of the mycelium. A detailed account of this study will be published in the future.

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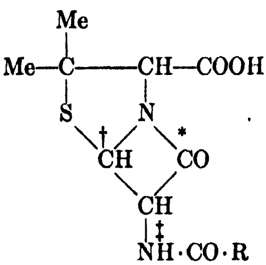
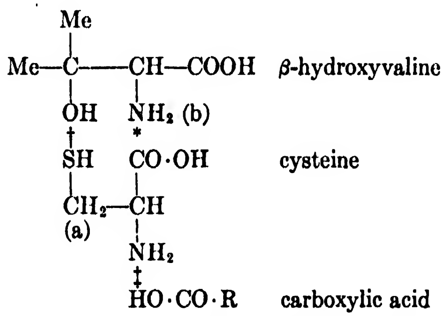
The Biosynthesis of the Penicillins

The following considerations have led the authors to suggest that the penicillin-type molecule (I) (1), is formed in *Penicillium notatum* from the amino acids, L-cysteine and D-β-hydroxyvaline, and a carboxylic acid (II).



Penicillin

I



Suggested as precursors of penicillin

II

The formation of a sulphide linkage between the mercapto acid, cysteine, and the hydroxyacid, β-hydroxyvaline, could be compared with the synthesis of cystathionine from cysteine and homoserine effected by *Neurospora* (2).

The assumption may, therefore, be made that the two amino acids, either free, or as part of larger peptide or protein molecules, may form the sulphide bridge at † and the peptide link at *, with further ring closure involving loss of one H atom from (a) and one from (b) to give the dicyclic structure (the order of these processes being, as yet, unspecified). Experiments carried out by one of us (3) indicate that the acylation process at ‡ takes place after the synthesis of the dicyclic

portion, which latter appears to be associated with the cell-substance of the mold.

The formation of dimethylpyruvic acid from glucose by a related mold, *Aspergillus niger* (4), appears to indicate that the production of substances related to valine may readily take place in similar molds.

One of us (5) has observed that the production of dimethylpyruvic acid is stimulated by the presence of acetate in the mold medium. Acetate has also been found to increase the production of penicillins by *P. notatum* (6). These phenomena may be linked, in that a precursor of these products might be β-hydroxydimethylpyruvic acid.

Further work on the biosynthesis of dimethylpyruvic acid is proceeding in this laboratory.

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A Metabolic Relationship between the Aromatic Amino Acids

The present work involves a further investigation of a *Neurospora* mutant, C-86, previously mentioned by Lein, Mitchell and Houlahan (1) as one that can utilize anthranilic acid, indole, tryptophan, kynurenine, 3-hydroxyanthranilic acid, and nicotinic acid as supplements for growth. Mutant C-86, when crossed to a "wild-type" strain, was found to differ from this wild type strain, with respect to tryptophan biosynthesis, by a mutation at a single locus.

A number of compounds were tested for growth-promoting properties for this mutant. These included: 3,4-dihydroxyphenylalanine, anthranil, benzoic acid, aniline, *p*-aminobenzoic acid, formylanthranilic acid, isatoic acid, *cis*-cinnamic acid, *trans*-cinnamic acid, phloroglucinol,

phenylacetic acid, *p*-aminophenylacetic acid, β-phenylethyl alcohol, phenyl-DL-α-alanine, β-phenylethylamine, salicylic acid, coumarin, coumaric acid, 2-carboxyindole, 3-carboxyindole, cinnamaldehyde, phenylalanine, and tyrosine. Of these compounds, phenylalanine, tyrosine, and *trans*-cinnamic acid were active in promoting the growth of C-86. The relative growths of this mutant on supplements of tryptophan, indole, anthranilic acid, phenylalanine, tyrosine and *trans*-cinnamic acid are given in Table I.

TABLE I

The Relative Growths of Neurospora Mutant C-86 in the Presence of Various Supplements

(The mold weights are for 20 ml. cultures grown at pH 4.6 and 25°C.)

μM	Dry wt. of mold—mg.—3 days growth						
	Tryptophan	Indole	Anthranilic acid	Phenyl-alanine	Tyrosine	*Trans*-cinnamic acid	
0.1	11	7	8	0	0	0	0
0.2	15	18	13	2	1	±	0
0.4	20	29	24	5	3	1	0
0.8	27	36	36	12	4	2	2
1.4	35	41	42	18	8	1	5
2.0	36	36	42	21	14	1	6
2.0	35	30	40	27	20	0	7

[a] Growth of mutant C-86 on supplements of *trans*-cinnamic acid at pH 5.6 and 25°C.

Neurospora mutant, 40008, which utilizes anthranilic acid, indole or tryptophan for growth, cannot use either phenylalanine, tyrosine, or *trans*-cinnamic acid. Apparently, strain C-86 has a genetic block which occurs at a point earlier in a reaction series involving tryptophan than does the block in strain 40008. This would imply that phenylalanine, tyrosine and *trans*-cinnamic acid are involved in the biosynthesis of tryptophan prior to the formation of indole or anthranilic acid in *Neurospora*. Another *Neurospora* strain, E-5212, utilizes phenylalanine for growth but none of the other substances found to promote the growth of strain C-86.

The evidence presented suggests the possibility of a common precursor to the aromatic amino acids.

ACKNOWLEDGMENTS

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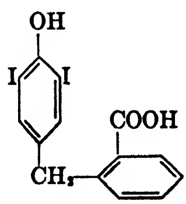
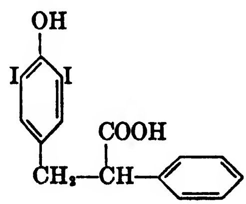
X-Ray Diagnostic Agents—2-(3,5-Diiodo-4-Hydroxybenzyl)-Benzoic Acid

In a recent publication, Jones *et al.* (1) have reported that, in the course of testing 2-(3,5-diiodo-4-hydroxybenzyl)-benzoic acid (I) as a cholecystographic agent in dogs, the extrahepatic biliary ducts were visualized along with the gall bladder. Since the inception of cholecystography in 1924, several iodinated aromatic acids (2) have been used or studied clinically as cholecystographic agents, and, without exception these compounds were found to be too toxic or exhibited untoward reactions such as diarrhea, nausea, and vomiting. In addition, poor absorption of several of these diagnostic agents resulted in interfering shadows in the cholecystograms.

As part of a comprehensive program on X-ray diagnostic agents, we prepared and tested I some time ago in view of its structural similarity to α-phenyl-β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid (II) (3). It is to be noted that II, and all of the other recently described iodinated compounds (4), suggested as cholecystographic agents are deriva-



I



II

tives of fatty acids. It has been assumed that the latter type of compounds have been satisfactory clinically as cholecystographic agents by virtue of the lipophilic character of these compounds and their sodium salts and their consequent property of being excreted into the bile and concentrated in the gall bladder. In view of the fundamental difference in the nature of the carboxyl group in compounds of type I and II, and the clinical history of type I compounds, it is of importance from the standpoint of the correlation of structure and cholecystographic property to determine the clinical efficacy of compound I.

In view of the pharmacological and clinical interest in I, we wish to record herein our synthesis of I, since Jones *et al.* (1) describe only the pharmacological data for this compound. The requisite intermediate, 2-(*p*-hydroxybenzyl)-benzoic acid (III) was obtained in 80% yield by the Raney reduction of 2-(*p*-hydroxybenzoyl)-benzoic acid. Iodination of III with potassium triiodide gave I in 83% yield.

Experimental

2-(p-Hydroxybenzyl)-Benzoic Acid (III)

To a solution of 20 g. of 2-(*p*-hydroxybenzoyl)-benzoic acid (5) in 350 cc. of 10% NaOH, there was added, with stirring, 30 g. of Raney's nickel-aluminum alloy (6) in the course of 1.5–2 hr. The reaction mixture, after heating for an additional hour, was filtered and the residual nickel catalyst washed with hot 2% NaOH. The combined filtrate and washing was acidified to Congo red paper with HCl, and, after cooling the acidified solution, the crude benzylbenzoic acid was filtered; yield 18 g., m.p. 130–136°C. Recrystallization from water gave a white product which melted at 148–149°C.; literature m.p. 145–146°C. (7).

2-(3,5-Diiodo-4-Hydroxybenzyl-Benzoic Acid (I)

To a solution of 22.8 g. (0.1 mole) of III in 800 cc. of 0.5 *N* NaOH solution, there was added dropwise, with stirring, a solution of 50.8 g. of iodine and 50.8 g. of KI dissolved in 250 cc. of H_2O. The iodinated mixture was stirred for 1 hr. at room temperature, filtered through Supercel, and the filtrate acidified with SO_2 until acid to litmus paper. The white crystalline solid was filtered, washed with water and dried; yield 40 g. (83%), m.p. 209–211°C. Recrystallization from ethyl acetate-petroleum ether or chloroform-petroleum ether gave I as a fine white crystalline material melting at 212.5–213.5°C.; literature 208–209°C. (1).

Anal. Calcd. for $C_{14}H_{10}O_3I_2$: I, 52.90; Neut. equiv. 240.
Found: I, 52.82; Neut. equiv. 239.

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A Role of Vitamin B_{12} in the Normal Mammal [1]

In continuance of previously reported work on an unidentified nutrient, X, for rats (1,2,3), we have now compared (Table I) crystalline vitamin B_{12} with APA 15 unit liver extract fed to weanling young as supplements to X-deficient basal rations (BR) adequate in respect to all chemically identified nutrients and containing 25%, 45%, and 65% of protein. The test young were reared by mothers that were on an X-deficient ration during the nursing period. In accordance with our previous findings, the 14 day growths on BR cotaining an X-deficient casein (casein C) decreased from 36 g. to 22 g. and 4 g., respectively, with these increases in percentage of protein. It is clear that supplementation with B_{12} increased the rate of growth on all the levels of protein, and that maximally effective doses of B_{12} produced practically the same effect as doses of liver extract which we have found to be maximally effective for male rats (*i. e.*, 0.05 cc. or 0.10 cc. of the brand of liver extract used). These doses of these supplements practically

[1] This project was supported by an allotment from Bankhead-Jones Special Research Funds.

TABLE I

Effect of Vitamin B_{12} on Growth

Protein in ration[a]		Sets of litter-mate male rats	Duration of assay	Negative control	Vitamin B_{12} (cryst.)		15 Unit parenteral liver extract	
Principal kind	Level			Av. gain in weight	Daily dose	Av. gain in weight	Daily dose	Av. gain in weight
	per cent		*days*	*g.*	γ	*g.*	*cc.*	*g.*
Casein C	25	10	28	64	0.01	86[b]	0.0025	120
					0.05	130		
Casein C	25	3	14	35	2.5	78	0.05	81
					5.0	78	0.10	85
Casein C	25	5	14	36	2.8	80	0.10	81
Casein C	45	4	14	22	2.8	74	0.10	72
Casein C	45	2	14	21	2.8	79	0.10	70
					5.6	70		
Casein C	65	2	14	4	5.0	53	0.10	55
Casein C	10	5	28	28	0.5	59		
Egg albumin	10	5	28	39	0.5	75		
Cottonseed	10	5	28	41	0.5	84		
Cottonseed	25	2	10	7[c]	0.5	42		
Soybean	25	2	10	5[c]	0.5	60		

[a] The basal rations (BR) consisted of casein C (extracted with 10 lots of hot alcohol, 6 hr. with each lot) or soybean oil meal to furnish the principal protein as indicated, dried yeast 10 ($\simeq$ protein 5), cottonseed oil 9.85, Navitol 0.15, salts 4.5, and (A) lactose 15 plus added vitamins (thiamine, riboflavin, pyridoxine, Ca pantothenate, niacin, inositol, PABA, biotin, pteroylglutamic acid, ascorbic acid, α-tocopherol acetate, and menadione) and dextrin to 100 or (B) dextrin to 100 without lactose or or added vitamins. No yeast was fed in the deglanded defatted cottonseed flour, heat-coagulated egg albumin or 10% casein basal rations; the vitamins were supplied by the mixture given above.

[b] t for difference from negative controls = 5.4**. ** Adjacent to a t value denotes a probability at the 1% level or beyond; * a probability of at least the 5% level.

[c] Weight gains of B_{12}-fed rats for the week before feeding this vitamin.

doubled, tripled, and increased 13-fold the rates of growth, respectively, on the 25%, 45%, and 65% protein rations. The growth rates on similar rations containing 10% of various proteins were likewise increased by supplementation with B_{12}, as had also occurred with our 10% protein ration containing casein C when supplemented with liver extract (1). Similar rations containing oil meals as sources of protein were evidently deficient in B_{12}, the growth rates of rats that survived on such rations containing 25% of protein increasing several-fold upon

supplementation with this vitamin. The increased growth rates on all of the B_{12} or liver extract-supplemented rations involved increases in food consumption.

The data in Table II show that the kidneys of similarly prepared rats maintained on an unsupplemented B_{12}-deficient 25% protein ration became hypertrophied; no definite pathology was detected histologically in these kidneys.

TABLE II

Effect of Vitamin B_{12} Deficiency on Kidney Weights

The basal ration contained 25% of protein (20% casein C, 5% yeast protein). Approximately maximally effective dose of 15 unit APA liver extract was the source of B_{12} activity.

Period on ration	Pairs of sex-litter-mate rats	Av. body wt. when sacrificed		Average weight of kidneys		
		No B_{12}	B_{12}	No B_{12}	B_{12}	*t* for diff.
days		*g.*	*g.*	*g.*	*g.*	
			Males			
28–36	6	142	228	2.43	2.55	0.8
54–70	26	215	322	2.97	2.79	2.3*
201–307	9	317	411	4.64	3.27	3.9**
			Females			
28–36	6	99	150	1.58	1.68	0.8
56–67	10	163	214	2.11	1.79	5.0**

(Males on stock colony diet: 4, approximately 270 days on diet, av. body weight 366 g., av. kidney weight, 2.79 g.; 2, approximately 2 years on diet, av. body weight 423 g., av. kidney weight 3.28 g.)

It is clear that, with all the levels of protein tested, a deficiency of B_{12} has a very deleterious effect on growth; we have reported that; with high levels of protein, such a deficiency may even be fatal (2), and we have found with such diets that, over a large range of growth levels, the B_{12} required to attain a given level of growth is increased. We believe that the above results indicate that vitamin B_{12} plays a fundamental role affecting the capacity of the normal mammal to utilize protein.

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Book Reviews

Advances in Food Research. Vol. I. Edited by E. M. Mrak and George F. Stewart. Academic Press, New York, N. Y., 1948. 459 pp. Price $7.50.

The rapid improvement and expansion of food research in universities and in the food industry has quite naturally been accompanied by a rapid improvement and expansion of the literature on food research. A welcome addition to this literature is the new annual series of reviews edited by Dr. Emil Mrak of the University of California and Dr. George F. Stewart of Iowa State College. Food research deals with the complex problems of delivering to the consumer the most nutritious and desirable foods at the lowest possible prices. These problems of the ancient art of food production and preservation are today being attacked by the common modern methods of biochemistry, physiology, and technology. The technological or engineering approach is largely neglected in this first volume of *Advances in Food Research*—a neglect which no doubt will be remedied in future volumes—and the first volume contains surprisingly little material that would seem out of place on the pages of *Archives of Biochemistry*. In the space of a short review, the individual articles of *Advances in Food Research* can be described only briefly.

The nutritive value of the original unprocessed foodstuff is influenced greatly by growing conditions, as is shown by the review of Somers and Beeson on "The Influence of Climate and Fertilizer Practices upon the Vitamin and Mineral Content of Vegetables."

Once food is grown, it must be preserved by some technique, such as cold storage, addition of chemical preservatives, canning or drying. All but two of the remaining reviews are concerned directly or indirectly with these various methods of preservation. Two reviews deal with fresh meat and its cold storage. Bate-Smith discusses "The Physiology and Chemistry of Rigor Mortis, with Special Reference to the Aging of Beef." This review emphasizes the relations of the practical problems of the storage and aging of beef to the present knowledge of muscle chemistry. Lowe discusses "Factors Affecting the Palatability of Poultry, with Emphasis on Histological Postmortem Changes." The work of Lowe is notable for the effective use of taste panels and of histological techniques in addition to the usual chemical methods, in the study of the quality of poultry meat and of the changes in quality on storage.

In his review on "Microbial Inhibition by Food Preservatives," Wyss discusses in terms of modern biochemistry the mechanisms of the actions of many chemical food preservatives. The review by Clifcorn, on "Factors Influencing the Vitamin Content of Canned Foods," makes clear the sincere and organized effort now being made to learn the effects of canning under different conditions on the vitamins of canned foods and to learn how to preserve the vitamins to the greatest extent possible. During the war, dehydrated foods, which could be shipped in compact form and stored without refrigeration, were of great military importance. The work on two dehydrated foods,

which were of particular military importance, is reviewed in this volume. Lightbody and Fevold discuss dried eggs and Ross discusses dehydrated potatoes. Both the initial quality and keeping quality of these products were very much improved by the war-time investigations. Dried eggs were finally produced for the Army which, even after long storage, could be made into scrambled eggs and omelets of first class quality.

A main cause of deterioration of the war-time dehydrated foods during preparation and storage was non-enzymatic browning, now recognized as one of the major reactions for good and for bad with which the food industry has to deal. Stadtman's review discusses the investigations of browning in fruit products, investigations which have been of great value for the understanding of non-enzymatic browning in general. The three reviews on dehydrated foods which have just been mentioned are based on the intensive work supported by the Quartermaster Corps, with which the Editors of *Advances in Food Research* were, and still are, intimately connected.

The quality of a foodstuff depends not only on its chemical composition but also on its physical character. The physical character of a large group of fruit products is achieved by the use of the jelling agent, pectin. "High-Polymer Pectins and Their Deesterification" are discussed by Baker. The whole subject matter of this review is typical modern polymer chemistry and enzyme chemistry.

Finally, we come to the acceptability of the food which has been produced and preserved by one method or another. The review by Lepkovsky discusses "The Physiological Basis of Voluntary Food Intake." The question of appetite is presented by Lepkovsky, not from the common psychological point of view, but from the point of view of the chemical composition and nutritive value of the food eaten and of the physiological state of the animal doing the eating.

All told, a good beginning in the creation of a review literature in the field of food research, although it must be said that some of the reviews are not too well written. But good writers of reviews are rare. In particular, it seems that authors do not find it easy to write reviews which are of detailed interest to the expert, and at the same time, permit the non-expert, without great effort, to get a rapid grasp of the field being reviewed.

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Biochemical Society Symposia. Committee of Publication for the Biochemical Society: J. H. Bushill, H. A. Krebs, E. J. King and R. T. Williams. No. I. The relation of optical form to biological activity in the amino acid series. A symposium held at University College Hospital Medical School, London, on February 15, 1947. Organized and edited by R. T. Williams. Cambridge. At the University Press. 1948.

This publication is devoted to the properties, especially biological ones, of the optical forms of amino acids. It deals essentially with L-forms and D-forms of α-amino acids. A. C. Chibnall discusses their nomenclature, which already has been established for a number of years, in the introduction. In the first place, the directional rotation in water of the individual amino acid was the criterion for the terms L- and D-form, respectively. After it had been established that all pertinent building blocks of albumin, according to their configuration, belong to the L-series, this property was no longer used; the L-form rather indicates that it belongs to the L-configuration in

general. This conclusion facilitates not only the classification of the α-amino acids to the natural or synthetic amino acid series; moreover, it is necessary for the recognition of polypeptides containing amino acids. It has been widely accepted that the various directional rotations of the individual amino acids be expressed within parentheses. Thus, L (+)-alanine. The necessity for further agreement in the classification arose with the discovery of those related to carbohydrates.

H. A. Krebs describes the status of our knowledge of L- and D-amino acid dehydrases (oxydases). The fundamentals of our knowledge we owe to Neubauer, and primarily to Knoop. It is well known that Emil Fischer's hypothesis, according to which only L-α-amino acids occur in albumin, in either the organism or the cell, has been disproved. The D-α-amino acids are present especially in microorganisms. It also has been established that polypeptides, which contain amino acids, are hydrolyzed by polypeptidases from vegetable and animal tissues. Great astonishment was caused by the finding that D-amino acids of animal organisms are broken down in addition to L-amino acids. The mere fact that, after feeding of DL-amino acids—depending in extent on their composition—not 100% of the administered D-form appeared in the urine, as was to be expected, indicated that the synthetic form of amino acid was being metabolized. An attempt to isolate a D-amino acid oxidase was successful. The corresponding L-enzyme system was established beyond doubt considerably later. Both kinds of enzymes differ substantially in their properties, but not in the mechanism of their action. O. Warburg and Negelein were able to crystallize the D-form as the Ba salt. Even before, the structural form of the enzyme had been recognized (flavinadeninedinucleotid proteid). It is of greatest interest that the activity of maximally purified L-enzyme, extracted by Green, falls far behind that of fresh tissues. The L-amino acid oxidase attacks the greater part of the L-α-amino acids. The hydroxyamino acids serine and threonine, the dicarboxylic acids asparagine and glutamine, and the diamino acids lysine and ornithine, do not break down.

Krebs reports on the occurrence of α-amino acid oxidases, their specificity, their properties, *etc.* There are also divers other findings which require further research. This, for instance, holds true for the statement that the enzyme obtained from the tissues of various vertebrates does not appear to be identical.

E. A. Zeller obtained especially effective amino acid oxidases from the secretions of venom glands of various poisonous snakes. Krebs also mentions the fact that enzymes of a special sort are effective for many amino acids: reduction of histidine, arginine, cysteine, phenylalanine, and tyrosine.

An excellent critical survey of the problem of the presence of D-glutamine acids in products of the hydrolysis of proteins of malignant tumors by means of strong HCl is given by G. R. Tristram. New methods for the demonstration of amino acids have been developed: chromatographic methods, methods of isotope dilution, electrodialysis, utilization of microorganisms, particularly for the differentiation of the L- and D-forms of amino acids.

Mention is also made of the possibility of the formation of steroisomers according to Walden's *Umkehrung.* It might be added here that Walden himself thought that *Waldenases* might be present in the cells. There are no indications at this stage of investigation about the presence of D-glutamine acids in the albumin of malignant tumors for Kögl's concept of the unique position of tumor proteins in regard to their

structure. Of course, there may be peculiarities in the sequence of the building blocks of proteins. It is surprising that no one points out the findings of Kögl and his coworker, von Erxleben, in which they list individually in tabular form the extablished amino acids. Besides glutamic acid, other amino acids, notably hydroxyglutamic acid, are cited. Their discoverer, Dakin, himself doubted their existence. Several scientists reported previously that the yield of this amino acid is minute or even zero. Kögl subjected relatively little amounts of protein to hydrolysis.

A. Neuberger investigates exhaustively the metabolism of D-amino acids in the organisms of mammals. The tests of feeding of DL-, L-, and D-α-amino acids have been mentioned previously. Numerous attempts of this kind also have been carried out on human beings. The occurrence of D-pyrrolidone carboxylic acid in the urine of rats, who were fed D-glutamine acid, is of great interest. It is pointed out that carnivores are better equipped with amino acid ureoxidase than herbivores. A report on the relation of amino acids which are foreign to the animal organism follows (Neubauer, Knoop): formation of α-keto acids. Entirely new is du Vigneaud and Irish's proof of the transformation of an amino acid into an acetyl derivative of the L-form. The accepted view is that of the dehydration of D-amino acids with formation of the corresponding α-keto acid and asymmetrical reamination accompanied by simultaneous acetylation. Additional investigations with labeled isotopes confirmed this reported finding also with other amino acids.

There follows a survey of the influence of certain D-amino acids on growth. We have reports on experiments with rats, mice, and chickens, the results being assembled n tabular form. In every case a check is made on whether transfer of a D-form to the corresponding L-form can be considered. The experiments with cystine and methionine to determine the effects of growth are of great interest. Derivatives and stereochemical forms were used for comparison (du Vigneaud). The relation of L-serine and L-homocysteine, derived from L-cystathionine, merits special consideration. A report on the excretion of special derivates of D-cysteine follows. Transformations which were established are determined in the combination and properties of mercapto acid, which is formed after feeding of aromatic-aliphatic halogen compositions.

The author deals especially with the relation of aromatic amino acids in the animal organism (tryptophan, phenylalanine, and tyrosine). The transformation of the first to *p*-hydroxyphenylalanine, and the manner of reduction 2,5-dihydroxyphenylalanine has been known for a long time. The breaking down of dopa is also mentioned.

In his contribution on amino acids and microbiological chemistry H. N. Rydon gives an excellent survey of the relation of the above mentioned amino acids in the metabolism of microorganisms, a field in which substantial progress has been achieved.

T. S. Work describes the D- and L-amino acids as antibiotics. These chemical substances, formed by microorganisms, are capable of inhibiting the growth of other species.

Work also discusses the problem of nutrition of bacterial cells in connection with the permeability of the cellular border. One always finds results of fundamental importance in the course of these discussions. At present, it is *Neurospora*, the study of which has led to many a new finding on enzymatic cell processes.

The study of biological properties of various amino acids and the products of their transformation—particularly the special composition of polypeptides—raises the

question, on which F. Bergel reports, whether D- and L-amino acids and their derivatives show special pharmacological effects.

Other problems dealt with include amino acids and calcium metabolism—calcification (H. Lehmann); relation of L- and D-form of amino acids to the growth of anaerobic molds (G. M. Hills). M. V. Tracey's note on the possible significance of amino acids concludes the work. He wonders whether, for instance, the configuration of amino acids might influence the enzymatic reduction of polypeptides—for example, in a protective sense. Scientists were already dealing with this question many years ago.

Altogether the Symposium affords a good survey of our present knowledge in the field of amino acids of the L- and D-series, also with reference to their relation in the organism.

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Topics in Physical Chemistry. A Supplementary Text for Students of Medicine. By W. MANSFIELD CLARK, DeLamar Professor of Physiological Chemistry, The School of Medicine, The Johns Hopkins University. Williams & Wilkins Co., Baltimore Maryland, 1948. xv + 738 pp. Price $10.00.

Many teachers in schools of medicine realize that explanations of physiological phenomena, and of important laboratory methods, are often to be found in the principles of physical chemistry. Yet the study of physical chemistry is not required of students preparing to enter medical schools, and there is little time for this subject in courses in medical biochemistry. As a result of this situation, Dr. Clark has prepared this book, not as a text for a formal course, but "to be drawn upon as the student of elementary biochemistry and the maturing student of medicine may find occasion."

This book is considerably longer than previous textbooks of physical chemistry for medical students, but the treatment is, in general, elementary. For the benefit of students without experience in quantitative chemistry, Dr. Clark has included chapters on the use of the analytical balance and on the exact measurement of volumes. Near the end of the book he gives a good deal of space to the optical instruments used in biochemical laboratories. The major portion of the text consists of a presentation of the principles of classical physical chemistry, with particular emphasis on their biochemical applications. Pertinent references to the literature are given directly in the text rather than in footnotes or in a bibliography.

The outstanding feature of the book is the skillful way in which the author has introduced applications to show the practical value of the principles. In the chapter on the gas laws, he describes the manometric apparatus used in the study of tissue metabolism. In the chapter on electrolytic conductance, he includes a method for estimating the "total base" of serum from its conductance. A large part of the chapter on mass-action equilibria is devoted to the reversible reaction between hemoglobin and oxygen. Other subjects of biochemical interest, not usually treated in books on physical chemistry, are discussed in the chapters on phenomena associated with membranes, protein solutions, and blood-electrolytes. Perhaps the most difficult chapters are those on free energy and oxidation-reduction potentials. The chapter on equilibria in proton exchanges is unusually good, with its account of titration curves, buffer action, and acid-base indicators.

This book, like Dr. Clark's previous writings, bears witness to his sound scholarship and his practical turn of mind. The reviewer is glad to recommend it to teachers and research workers in biochemistry as well as to students of medicine.

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Vitaminas, Metodos de Dosificacion. By GILBERTO G. VILLELA, Chief of the Chemical and Pharmacological Division of the Oswald Cruz Institute in Rio de Janeiro, and lecturer in Biochemistry at the Medical Faculty of the University of Brazil. With an introduction by Dr. Venancio Deulofeu, Professor of the University of Buenos Aires. Translated from Portuguese into Spanish. El Ateneo, Buenos Aires, 1948. xvi + 442 pp. (bound in paper).

Latin-America and the entire Spanish speaking world is to be congratulated on Gilberto G. Villela's "Methods of Vitamin Determinations." Strange as it may seem, there is no counterpart of this book in the English language. The subject of vitamin analysis has been treated quite differently in "Estimation of the Vitamins," edited by W. J. Dann and G. H. Satterfield (*Biological Symposia*, **12**, 1947) and "Methods of Vitamin Assay," by the Association of Vitamin Chemists (Interscience, 1947). While the latter gives exact procedural details on some selected methods for a few of the vitamins, the former is a collection of essays on procedures presented in different ways by a number of different authors. Villela's book lists quite evenly all of the important, and some of the less established, procedures, usually in a form sufficiently detailed to allow a man trained in the field of analysis to carry out the determinations without much difficulty.

Villela discusses each vitamin in a separate chapter. The fat soluble vitamins A, D, E, and K are followed by vitamin C and the B complex, thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, biotin, *p*-aminobenzoic acid, choline, inositol, and folic acid. There is no systematic discussion on provitamin D analysis and there is no discussion on vitamin P or the analysis thereof.

The arrangement of each vitamin chapter may best be illustrated by describing one of the chapters more fully: Nicotinic acid and nicotinamide cover 33 pp. in this volume. This section begins with a list of the names given to this vitamin, the empirical and structural formulae (the ring nitrogen has been left out of the formula for nicotinic acid), followed by an historical "introduction" and a presentation of the "physicochemical properties and characterization." Paragraphs on "toxicity" and "distribution" in nature are followed by "requirements" where the data recommended by the Food and Nutrition Committee of the National Research Council of the U. S. are given. "Metabolism," "coenzymes I and II," and a discussion of the presence of the nicotinic acid molecule in urine and in blood, is followed by a short paragraph on pellagra and nicotinic acid deficiency symptoms. All this takes up one-third of the total space devoted to this vitamin. Coming to the methods of nicotinic acid determination, Villela discusses first the bioassay on dogs. Very appropriately he recommends the procedure used by Waisman and Elvehjem.[1] W. J. Dann in the "Estimation of the Vitamins" does not refer to this method, but to a later paper by Schaefer,

[1] Waisman, H. A., and Elvehjem, C. A., The Vitamin Content of Meat. The Burgess Publishing Co., Minneapolis, Minn., p. 121, 1941.

McKibbin and Elvehjem[3] which, in the eyes of the investigators, describes perhaps a more reliable method, although this procedure has not been subjected to sufficiently extensive tests to judge the precision. Bioassay for nicotinic acid activity has recently been found indispensable in spite of all the other methods available. The problem of fortifying rice and grits with the various B vitamins called for a water-insoluble form of nicotinic acid. The physiological activity of any such compound can be tested only on dogs. Therefore, Villela did well to include the bioassay on dogs.

A discussion of the well-known chemical assay procedures, the cyanogen bromide method and the 2,4-dinitro-chlorobenzene method follow. There is also a paragraph on the fluorometric determination of nicotinamide in the presence of nicotinic acid, and there are discussions on the modifications of the basic techniques for the determination of nicotinic acid and derivatives in urine and in blood. Finally there is a description of microbiological assay procedures. The *Lactobacillus arabinosus* test is described for routine assays and the test using *Proteus vulgaris* for nicotinamide in blood. The chapter ends with a discussion of the methods available for the determination of nicotinic acid deficiency in man. There are 66 references to original literature in this section, plus numerous other references by author's name only.

The chapters on the other vitamins are arranged in the same systematic fashion. The selection of the specific modification for each method discussed, and the presentation of the general method, is perhaps not always quite as critical as one might expect for a treatise on analyses. One might also wish to find more information on the limitations of the various assays and on the sensitivities to be expected from each procedure.

Generally speaking, this reviewer, who does not read Spanish very fluently, is under the impression that the various chapters have been prepared with about equal care. The chapter on vitamin D is perhaps the weakest because it contains so many statements which are not absolutely accurate: the provitamin D in human skin has not been identified (p. 101), nor has the involvement of the sebaceous gland been proven (p. 101). It was not at the 1931, but at the 1934, Conference that the relation of the biological activity of the irradiated ergosterol solution to crystalline vitamin D_2 was defined, *etc.* Particularly puzzling to this reviewer is the table on p. 100 listing the relative biological activities of the vitamins D_2, D_3, D_4, and D_5 for chicks and rats. Vitamin D_2 is given a value of 1,000 for both species, D_3 shows 25,000 for chicks and 1,000 for rats, D_4 (which is not otherwise mentioned or identified in the text) rates 2,000 for chicks and 100 for rats, *etc.*

This book will surely find a wide and well deserved distribution in the Spanish speaking countries. To the English reading public, however, this book is not a "must" in spite of the fact that a few original contributions are included, such as the fluorometric determination of folic acid solutions. But to brush up on scientific Spanish Villela's book will prove interesting reading.

H. R. ROSENBERG, New Brunswick, N. J.

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[3] Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.* **144**, 679 (1942).

Recent Progress in Hormone Research. The Proceedings of the Laurentian Hormone Conference, Vol. III. Academic Press, Inc., New York, N. Y., 1948. viii + 379 pp. Price $7.80.

The third volume of the proceedings of the Laurentian Hormone Conference is a good demonstration of the success of this meeting. Twelve papers were presented by authorities in their respective problems and the ample discussion enhances the value of the published reports. The problems considered were grouped in 5 main subjects.

The first paper is on the biochemistry of pituitary growth hormone by Choh Hao Li and H. M. Evans. The first demonstration of growth interruption by hypophysectomy (Caselli, 1900) was followed by the demonstration (Evans and Long, 1921) that the injection of anterior pituitary can accelerate the growth rate of the rat and produces gigantism. The persistent researches of this laboratory were climaxed when the growth hormone was isolated in pure form by Li *et al.* (1944–1945). In their paper the authors describe the physical properties, the chemical composition and the biological action of the growth hormone: (1) increase of growth; (2) lowering of blood amino acids; (3) nitrogen retention; (4) elevation of alkaline phosphatase level in the plasma; (5) increase in weight of liver and thymus.

In the second section on "Steroid Hormones," 3 papers were presented. The first is by K. Miescher, on the "Relation of Activity to Constitution of Sexogens," with special reference to doisynolic acid. This substance was obtained by opening the keto ring of the estrogens and has a high activity on oral administration. The second paper, of S. Lieberman and K. Dobriner, is an authoritative contribution to the important problem of identification of steroids excreted in urine in health and disease. The third paper, by H. L. Mason, is a description of the identification and titration of many interesting urinary steroid compounds excreted in cases of adrenal disease, adrenal cortical tumors and cortical hyperplasia, observed by Kepler and Sprague at the Mayo Clinic.

The histochemical and histophysical methods in hormone research were discussed in 2 papers. E. W. Dempsey emphasizes the difficulties of the problem in his review of the procedures now available for characterizing the chemical organization of the cells of the endocrine glands. In a short and clear paper, C. P. Leblond gives a summary of his work, using the radioiodine that has made it possible to trace the behavior of iodine from the time it enters the body through its transformation into thyroid hormone and the excretion of this hormone in the feces.

The physiology and function of the testis is presented in 4 papers and was discussed from many angles and by many specialists in the experimental and clinical fields. C. W. Hooker contributed his own research and a discussion of a part of the complex and debated problem of the biology of the interstitial cells of the testis; there is no mention of the classic and fundamental historical papers (Ancel, Borun, *etc.*). W. O. Nelson and C. G. Heller emphasize the possibility of diagnosing and classifying the various types of hypogonadism in male patients on the basis of physical findings, testicular biopsy and assays of urinary gonadotrophins.

The paper on the testis-pituitary relationship in man by C. G. Heller and W. O. Nelson was supplemented by lengthy discussions and factual contributions of many specialists in the field. The paper of J. B. Hamilton on the role of testicular secretions as indicated by the effects of castration in man and by studies on pathological condi-

tions and the short lifespan associated with maleness, is a contribution full of excellent information.

The fifth section discussed was "Hormones and Hypertension." The paper by A. C. Corcoran on renal pressor system and experimental and clinical hypertension is a masterly presentation and a good resume of the American literature, and especially of the personal experience and opinions of the author. The paper of Hans Selye is an excellent exposition of his experimental work and his theoretical interpretations of the diseases of adaptation described by him.

B. A. Houssay, Buenos Aires.

Distribution of a Triphosphopyridine Nucleotide-Specific Enzyme Catalyzing the Reversible Oxidative Decarboxylation of Malic Acid in Higher Plants [1]

Eric Conn, Birgit Vennesland and L. M. Kraemer [2]

From the Department of Biochemistry, University of Chicago, Chicago, Illinois

Received March 14, 1949

The widespread distribution of the enzyme oxaloacetic carboxylase in the tissues of higher plants has recently been demonstrated (1). The reaction catalyzed may be written:

$$\text{oxaloacetate} \rightleftarrows \text{pyruvate} + CO_2. \qquad (1)$$

Previous work of Ochoa, Mehler and Kornberg (2), on a similar enzyme in pigeon liver, has indicated the existence of a close relationship between the enzymic decarboxylation of oxaloacetic acid (OAA) and the oxidation of malic acid by triphosphopyridine nucleotide (TPN). The purified "malic enzyme" of pigeon liver catalyzes an overall reaction which may be written:

$$l\text{-malate} + TPN_{ox} \rightleftarrows \text{pyruvate} + CO_2 + TPN_{red}. \qquad (2)$$

Evidence has previously been published for the presence, in an OAA carboxylase preparation from parsley root, of a TPN-specific enzyme which catalyzes the reversible oxidative decarboxylation of malic acid (3). The experiments described in the present paper constitute an examination of the question whether this enzyme can always be demonstrated in OAA carboxylase preparations from higher plants. For sake of brevity, this activity will also be referred to as a "malic enzyme," although further work is necessary to establish whether the

[1] This work was supported in part by grants from the John and Mary R. Markle Foundation and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[2] Department of Chemistry, University of Chicago.

mechanism of the reaction in plants is identical with that in pigeon liver. During this investigation it became apparent that the widespread occurrence of enzymes which inactivate TPN seriously interfered with the demonstration of the malic enzyme. When the assay procedure was designed to avoid this difficulty, it was possible to show the presence of the malic enzyme in all of the plant carboxylase preparations tested.

Materials and Methods

The protein preparations employed in this work all contained oxaloacetic carboxylase. A summary of the methods of preparation, and of the carboxylase activity of the various crude enzymes used, is given in Table I.

TABLE I

Description of Plant Preparations

Sample	Carboxylase activity *K/g. protein*	Dry weight *mg./ml.*
Wheat germ, Type A	35	19
Wheat germ, Type B	214	69
Wheat germ, Type C	326	113
Beet	32	14
Carrot	87	4
Parsnip	22	27
Parsley root	75	10
Spinach	4	20
Peas	11	35

The Type A wheat germ, beet, carrot, and spinach preparations were the fractions obtained by precipitation with $(NH_4)_2SO_4$ in the concentration range of 250–500 g./l. of extract or press juice (1). The parsley root and parsnip preparations were dialyzed solutions of the lyophilized preparations previously described (3).

Type B wheat germ: Water extract (4 ml./g. wheat germ) was fractionated with solid $(NH_4)_2SO_4$ at pH 6.5, retaining the precipitates, successively, between $(NH_4)_2SO_4$ concentration limits of:

1. 185 and 365 g./l.
2. 100 and 240 g./l.

Type C Wheat Germ: Procedure was that followed with Type B, the concentration limits being:

1. 190 and 365 g./l.
2. 240 and 340 g./l.
3. 290 and 340 g./l.

The pea preparation, kindly furnished by M. B. Mathews of the Dept. of Chemistry was a sample enriched in DPN-formic dehydrogenase. It was precipitated by $(NH_4)_2SO_4$, within the concentration limits of 220 g./l. and 330 g./l., from an extract of dried green peas (equal weights of water-soaked peas and 0.1 M Na_2HPO_4).

In all cases where $(NH_4)_2SO_4$ precipitates were used, care was taken to remove the salt completely by dissolving the precipitate in 0.025 *M* phosphate buffer, pH 7.3, and dialyzing against water or buffer. Any residue remaining was then centrifuged and discarded.

The carboxylase activity is expressed in term of K/g. dry weight of protein, where K is the monomolecular reaction rate constant in reciprocal minutes. Calculations of specific carboxylase activity have been described elsewhere (3).

Yellow enzyme was prepared from brewer's yeast[3] according to the method of Warburg and Christian (4). The partially purified material used in these studies was that obtained by precipitation with methanol and drying over H_2SO_4 at 0°C. The yellow powder was dissolved in water and centrifuged; the clear solution was then used in the test system in amounts known to be in excess. The absorption coefficient of this material at 465 mμ was 9.2 cm.^{2}/g. This figure cannot be used to calculate purity from Theorell's (5) value for the absorption coefficient, since impurities which absorb at 465 mμ are known to be present

TPN was prepared from beef liver by a modification of the procedure of Warburg, Christian and Griese (6). The purity of the material was determined by enzymatic reduction with *Zwischenferment* and glucose-6-phosphate (6) and by reduction with $NaBH_4$ (7). Using the value for β of 1.3×10^7 cm.^{2}/mole (8),[4] the TPN purity was found to be 15%. The purity of diphosphopyridine nucleotide (DPN) samples obtained from Schwarz Laboratories was determined by reduction with $NaBH_4$ and ranged between 50 and 60%. The DPN samples did not contain any TPN since the material was inactive in the specific manometric assay system for TPN described by Warburg *et al.* (6). The TPN did not contain appreciable DPN since the same amount of absorption at 340 mμ was obtained whether the sample was reduced by $NaBH_4$ (which reduces both DPN and TPN or whether is was reduced specifically by *Zwischenferment* and glucose-6-phosphate. When tested with the DPN-specific formic dehydrogenase of dried green peas (9), the maximum DPN content of the TPN preparation was determined to be less than 1%.[5] Yeast adenylic acid and adenosine were purchased from Schwarz Laboratories and muscle adenylic acid from the Sigma Chemical Company. *l*-Malic acid was purchased from Eastman Chemicals and recrystallized twice from ethyl acetate-petroleum ether before use. Glycylglycine was purchased from Pfanstiehl Chemicals, and catalase was the commercial product, Catalase-Sarett, sold by the Vita-Zyme Laboratories. Glucose-6-phosphate was synthesized from the monoacetone of glucose according to Levene and Raymond (10). All solutions were adjusted to pH 7.4 before use.

The manometric experiments were carried out in the Warburg apparatus at 37°C. Experiments measureng O_2 uptake were carried out in an atmosphere of 100% O_2. CO_2 evolution was measured according to the direct method described by Dixon (11).

[3] We wish to thank the Ambrosia Brewing Company of Chicago for a supply of yeast.

[4] $\beta = \dfrac{\ln I_0/I}{c \times d}$ (cm.^{2}/mole).

[5] Unpublished experiments of Mr. M. B. Mathews, Dept. of Chemistry, Univ. of Chicago.

The spectrophotometric measurements were carried out on a Model DU Beckman spectrophotometer.

Pyruvate analyses were made by the hydrazone method of Friedemann and Haugen (12), using both the direct and specific extraction procedures. Pyruvate was also analyzed by decarboxylation with yeast pyruvic carboxylase (13). Malate was determined according to Speck (14). Since high blanks were obtained in this procedure it was necessary to use an internal standard prepared by adding known amounts of malate to a reaction mixture which had been incubated without malate. Small amounts of TPN (1–15 γ) were determined manometrically by the *Zwischenferment* method of Warburg *et al.* (6).

Results

Direct Spectrophotometric Tests for TPN-Malic Enzyme

The spectrophotometric test employed by Ochoa *et al.* (2) could sometimes be applied very successfully to demonstrate the occurrence of Reaction 2 in various plant preparations. The test is dependent on the measurement of light absorption at 340 mμ, due to the reduced TPN, and has the advantage that the reversibility of the reaction can readily be shown. A typical experiment of this sort, with a wheat germ preparation, is presented in Fig. 1. The results are essentially similar

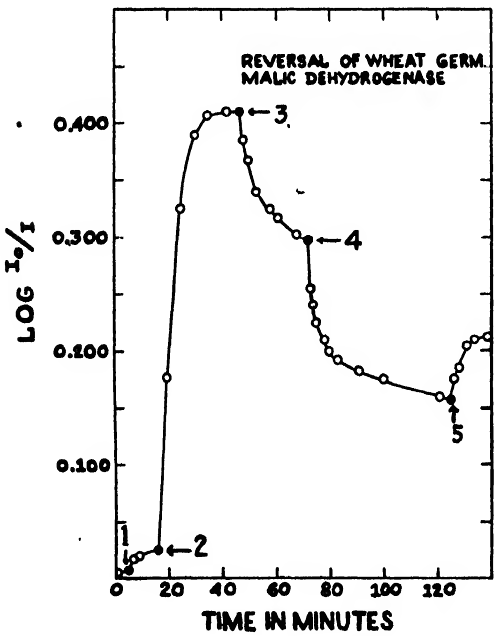

FIG. 1. Reversal of TPN-malic enzyme from wheat germ. Reduction and oxidation of TPN determined spectrophotometrically at 340 mμ. Corex cells, d = 1.0 cm. Temperature, 25°C. Measurements made against a blank which received all additions except TPN. Optical density corrected for changes on dilution. 1.1 ml. 0.1 M glycylglycine buffer pH 7.4; 240 γ TPN, and water made to a volume of 3.0 ml. At 0 time, 0.02 ml. 0.5 M l-malate added. At 1, 0.16 mg. wheat germ, Type C added. At 2, 0.1 ml. 0.01 M $MnCl_2$ added. At 3, 0.1 ml. 0.5 M pyruvate added. At 4, 0.1 ml. 1.0 M $NaHCO_3$ saturated with CO_2 added. At 5, 0.1 ml. 0.5 M l-malate added.

to those obtained with parsley root (3) and show the dependence of the reaction on the presence of Mn^{++} (Co^{++} may be substituted for Mn^{++}). Reoxidation of TPN_{red} by addition of pyruvate and bicarbonate is shown at times 3 and 4. The reduction obtained by addition of malate at time 5 further demonstrates the ease with which the equilibrium of the reaction can be shifted.

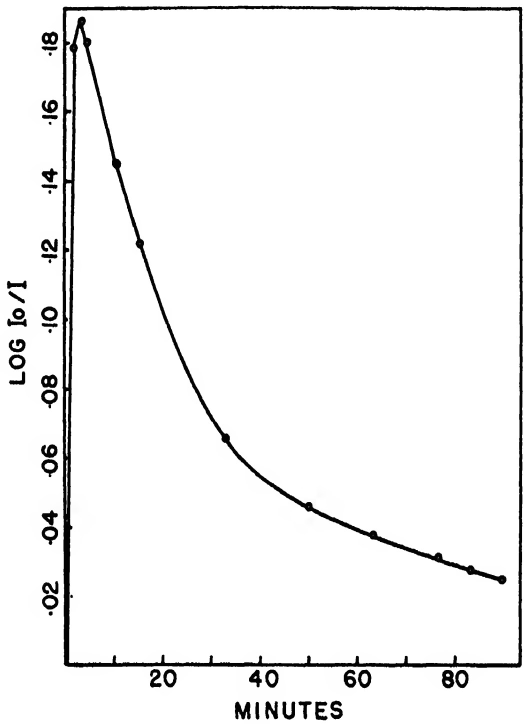

FIG. 2. Destruction of TPN by carrot enzyme. Reduction of TPN determined spectrophotometrically at 340 mμ. Corex cells, $d = 1.0$ cm. Temperature, 25°C. Measurements made against a blank which received all additions except TPN. 1.0 ml. 0.1 *M* glycylglycine buffer, pH 7.4; 2 mg. carrot enzyme; 0.1 ml. 0.01 *M* $MnCl_2$; 0.1 ml. 0.1 *M* *l*-malate; and water made to a volume of 2.9 ml. At 0 time, 120 γ TPN added.

Application of the spectrophotometric test to various OAA carboxylase preparations did not, however, give uniformly positive results for the presence of the malic enzyme. In some cases no reduction of TPN was observed, in others only a small and very slow reduction was obtained, and in still others the reduction of the coenzyme did not

appear to reach an equilibrium point but was followed by an apparent reoxidation, although no known oxidizing agent such as pyruvate or bicarbonate had been added. An extreme example of this latter sort, obtained with a carrot preparation, is shown in Fig. 2. The possibility of reoxidation of reduced TPN by pyruvate in the presence of lactic dehydrogenase could be eliminated since the preparation did not contain the latter enzyme. Analysis for TPN remaining at the end of the experiment showed that all the TPN had disappeared.

These and similar results with other plants suggested that TPN-inactivating factors were present in variable amounts in the plant preparations. One would fail to observe the presence of the malic enzyme if these factors destroyed the TPN before commencement of observations. Even in cases where the spectrophotometric test was used successfully (such as that shown in Fig. 1), it was possible to show that some inactivation of TPN occurred. Thus, the plateau reached preceding arrow 3 in Fig. 1 is only apparent. If sufficient time was allowed to lapse before addition of any oxidizing agent, a decrease in light absorption occurred.

Manometric Test System for TPN-Malic Enzyme

The malic enzyme could also be detected by a method which involved a substitution of malate and malic enzyme for glucose-6-phosphate and *Zwischenferment* in Warburg's old yellow enzyme system. In this procedure, TPN is reduced by malate in the presence of malic enzyme, and the reduced TPN is then oxidized by yellow enzyme which, in turn, is oxidized by molecular oxygen. The rate of O_2 consumption may, therefore, serve as a measure of the malic enzyme present provided the other components of the sytem are present in excess. A similar system has been used by Adler *et al.* (15) to study isocitric dehydrogenase.

A detailed study of this reaction sequence was made with the various wheat germ preparations which were available. The dependence of O_2 consumption on the presence of all components is shown in Fig. 3. Potassium cyanide (0.0025 *M*) was employed in this experiment, as it was in Warburg's *Zwischenferment* system, to inhibit catalase and thus prevent the breakdown of H_2O_2 formed in the reaction between yellow enzyme and O_2. The O_2 consumption of the complete system containing malate wheat germ, TPN, Mn^{++}, and yellow enzyme is shown in Curve 1. Curve 2 shows that, when KCN was omitted, the O_2 consumption was about one-half that observed in its presence. This would be expected if the peroxide formed were decomposed to H_2O and O_2 by the catalase known to be present in the Type C wheat germ sample used. Substitution of an excess of DPN for TPN resulted in a negligible O_2 uptake, as shown in Curve 3. A negligible O_2 uptake also resulted upon omission of yellow enzyme or TPN (Curves 4 and 5), and upon omission of Mn^{++}, malate, or wheat germ, or heat inactivation of the wheat germ enzyme (Curves 6–9).

Glycylglycine buffer was used in these experiments in preference to phosphate buffer since the latter precipitates Mn^{++} below its effective concentration and, therefore, causes some inhibition. Since a 10-fold increase in Mn^{++} concentration gave no increase in the oxidation rate, the concentration was assumed to be near optimum value. The malate concentration (0.0125 *M*) used in the experiment shown in Fig. 3

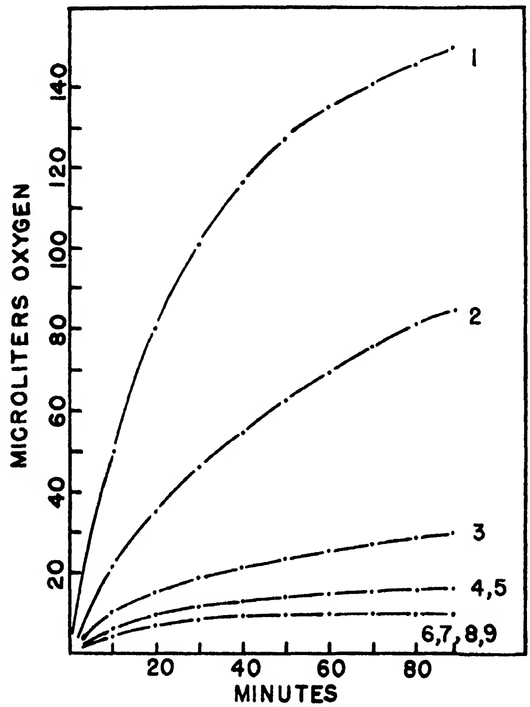

FIG. 3. Demonstration of TPN-malic enzyme in wheat germ. Complete system (Curve 1) contained 0.0125 *M* *l*-malate; 0.0025 *M* KCN (neutralized to pH 7.4); 0.05 *M* glycylglycine buffer, pH 7.4; 0.00025 *M* $MnCl_2$; 11.3 mg. wheat germ, Type C; 1.5×10^{-5} *M* TPN; 20 mg. yellow enzyme; and water to a total volume of 4.0 ml. Curve 2, KCN omitted. Curve 3, 5×10^{-5} *M* DPN substituted for TPN. Curves 4 and 5, yellow enzyme or TPN omitted. Curves 6, 7, 8 and 9, wheat germ enzyme heat-inactivated or $MnCl_2$, malate, or wheat germ omitted. Reaction started by adding yellow enzyme together with DPN when indicated from side arm after temperature equilibration. Gas, O_2; temperature, 37°C. KOH in center well.

was less than the optimum value. In most of the other experiments larger concentrations (0.062 and 0.083 *M* malate) near the optimum value were employed. An increase in the yellow enzyme concentration did not increase the rate of O_2 uptake.

Evidence that the reaction proceeds according to Eq. (3)

$$l\text{-malate} + \tfrac{1}{2}\,O_2 \rightarrow \text{pyruvate} + CO_2 + H_2O \qquad (3)$$

was obtained by measuring malate and O_2 utilization and pyruvate and CO_2 formation in the presence of added catalase and in the absence of cyanide.[6] It was hoped that this would ensure the decomposition of the peroxide formed and thus avoid any oxidation by the peroxide itself. However, as will be described later, this objective was not always achieved. Since some knowledge of the applicability of the manometric test system to malic enzyme preparations of varying degrees of purity was desired, 3 preparations of wheat germ were employed. Experiments were performed with both an excess and a limiting amount of malate and representative results are given in Table II. When an accurate measure of malate utilization was desired, small amounts of malate were used.

TABLE II

Balance Experiments with Wheat Germ

Type of preparation	Malate added initially	Malate found at end of experiment	O_2 used	Pyruvate formed	CO_2 formed
	micromoles	*micromoles*	*micromoles*	*micromoles*	*micromoles*
10 mg. Type A	10	5.4	6.0	5.3	7.0
3.5 mg. Type B	250	—	5.4	5.7	7.4
11 mg. Type C	250	—	3.5	6.0	7.3

All vessels contained 0.067 M glycylglycine buffer, pH 7.4, 0.033 M $MnCl_2$; 20 mg. yellow enzyme, 1.2×10^{-5} M TPN; 0.03 mg. catalase, water to 3.0 ml., and the amounts indicated of malate and wheat germ protein. Reaction was started by tipping in yellow enzyme and TPN from side arm after temperature equilibration. Gas, O_2; temperature 37°C. Reaction followed until O_2 uptake ceased, usually a period of 150-180 minutes. At this time, 2.5 ml. of the reaction mixture were removed and added to 2.5 ml. of cold 10% trichloroacetic acid. The resulting precipitate was centrifuged down and malate and pyruvate analyses by the hydrazone method were conducted on properly diluted aliquots of the supernatant. Pyruvate analyses by yeast carboxylase were done on aliquots of the untreated reaction mixture.

According to Eq. 3, the moles of malate removed should be equal to the moles of pyruvate and of CO_2 formed and twice as great as the moles of O_2 consumed. The data in Table II show that these expectations were always fulfilled for malate disappearance, pyruvate formation and CO_2 formation, but for O_2 consumption the expected values were obtained only with Type C preparation. In the case of Types A and B preparations, the O_2 consumption was twice the theoretical value. This is the amount which is expected if the peroxide were not decom-

[6] Cyanide can affect the reaction in 3 ways: by inhibiting catalase; by inhibiting peroxidase; and by binding OAA as the cyanohydrin. Cyanide effects of the type shown in Fig. 3 are not obtained with all preparations. A complete rationalization of all the effects observed has not yet been achieved.

posed to give H_2O and O_2. The preparations were found to contain an active peroxidase as tested by the method of Avery and Morgan (16). Furthermore, Keilin and Hartree (17) and Chance (18) have demonstrated that catalase may exhibit peroxidase activity under conditions where peroxide is generated slowly. It seems probable, therefore, that a catalyzed oxidation of an unidentified component of the system by peroxide occurred with preparations A and B, but not with preparation C. The possibility of its being glycylglycine may be discarded, as the same results were obtained with several other buffers, namely, veronal, tris[7] and ammonia. Although pyruvate is reported to react with H_2O_2 in bacterial preparations, even in the presence of catalase (19), no such reaction occurs in the plant materials under the conditions of these experiments, since pyruvate can be readily determined, by 3 different procedures, to be present in the amount expected.

Effects of Nucleotides other than TPN on the Manometric Test System

The rate of O_2 consumption observed in the manometric test for malic enzyme always showed a decline with time. The rapidity of this decline varied with different preparations tested. In all cases where the experiment was continued for a sufficient period of time the reaction eventually stopped. This effect was shown to be due to the destruction of TPN added, rather than to an inactivation of the malic enzyme.

The effects of ATP and DPN on the synthesis of TPN in pigeon liver as reported by Mehler *et al.* (20), as well as the work of Mann and Quastel (21), and of Kornberg and Lindberg (22), on the ability of nicotinamide to prevent DPN destruction suggested that these compounds might be effective in preventing the destruction of TPN. In addition, Ceithaml and Vennesland (23) had shown that ATP together with TPN increased the incorporation of labeled CO_2 into isocitrate by parsley root protein and suggested that ATP had a sparing action on TPN.

Examination of these various possibilities revealed that ATP, DPN, and muscle adenylic acid could all serve effectively to prevent the destruction of TPN in the wheat germ preparations. Results of a typical experiment are shown in Fig. 4. This particular experiment was designed to demonstrate the effects of DPN and ATP, and also to provide evidence that the differences in the rates are associated with the disappearance or maintenance of TPN. The first portion of Curve 4 in this figure is identical with Curve 1, Fig. 3. At 90 min., when the re-

[7] Tris(hydroxymethyl)-aminomethane, obtainable from the Commercial Solvents Corporation, 17 E. 42nd Street, New York, N. Y.

action had stopped, an analysis for TPN showed that none was present. The rate of the reaction was immediately restored almost to its initial level when more TPN was added to the reaction mixture, but again fell to a low value after 100 min. These findings, therefore, are in agreement with the previous observations that there is a TPN-inactivating factor in wheat germ. For the sake of brevity this destructive factor will be referred to as a TPN-ase but the name should not be understood to imply any mechanism of its action. Curve 6 of Fig. 4

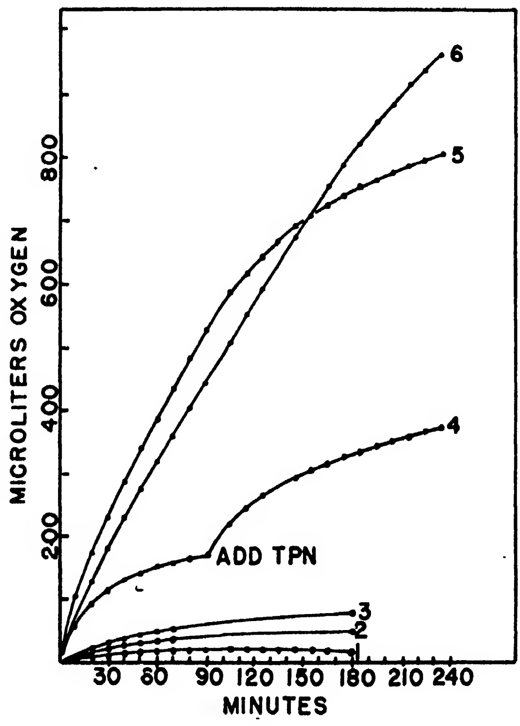

FIG. 4. Demonstration of TPN-malic enzyme, in wheat germ. Complete system (Curve 4) contained 0.0625 *M* *l*-malate; 0.0025 *M* KCN (neutralized to pH 7.4); 0.0625 *M* glycylglycine buffer, pH 7.4; 0.0025 *M* $MnCl_2$; 20 mg. yellow enzyme, 1×10^{-5} *M* TPN; 11.3 mg. wheat germ, Type C; and water to a total volume of 4.0 ml. At 90 min. on Curve 4, another 30 γ TPN added from a second side arm. Curve 1, heat-inactivated wheat germ enzyme employed or malate omitted. Curve 2, 1.5×10^{-4} *M* DPN substituted for TPN. Curve 3, 0.0015 *M* ATP substituted for TPN. Curve 5, 1.5×10^{-4} *M* DPN added to the complete system. Curve 6, 0.0015 *M* ATP added to the complete system. Reaction started by tipping in yellow enzyme and TPN together with ATP or DPN, when indicated, from side arm after temperature equilibration. Gas, O_2; temperature, 37°C. KOH in center well.

demonstrates that 6 micromoles of ATP greatly prolonged the period of rapid O_2 uptake, and at 90 min. an analysis for TPN showed that 90% of the TPN initially added could be recovered. It is clear, therefore, that ATP addition had prevented the destruction of TPN in this case. A similar effect of DPN is shown in Curve 5. The small differences between Curves 5 and 6 should not be interpreted as a measure of the relative effectiveness of the two compounds, since equimolar concentrations were not employed. Blanks obtained by omitting malate, or by substituting DPN or ATP for TPN are given as Curves 1, 2 and 3, respectively. These data were obtained with Type C wheat germ preparation. Qualitatively identical results are obtained with the Type A wheat germ.

Muscle adenylic acid (adenosine-5-phosphoric acid) gave an effect identical with that observed with an equivalent concentration of ATP. Yeast adenylic acid (adenosine-3-phosphoric acid) is about one-half as effective as muscle adenylic acid or ATP. Nicotinamide, adenine, adenosine, guanine, ribose, orthophosphate and pyrophosphate did not exhibit any sparing action on TPN destruction in the wheat germ samples.

The manometric test system for malic enzyme has also been applied to a dialyzed extract of pigeon liver acetone powder (24). Using 0.5 ml of the extract, 350 μl. of O_2 were utilized in 180 min. in the presence of 0.08 M malate, and only 15 μl. were used when malate was omitted. ATP and DPN addition increased the O_2 uptake somewhat, although not as much as in the case of wheat germ, the values being 520 μl. and 440 μl., respectively, when 0.002 M ATP or 2×10^{-4} M DPN are added. These results are in agreement with the observation that pigeon liver extracts destroy TPN only very slowly and, therefore, large stimulation effects of ATP or DPN should not be observed. In one respect, the pigeon liver preparation differs markedly from the plant preparations. 0.002 M ATP without TPN causes an O_2 utilization of 200 μl. in 180 min., corresponding to 57% of the value obtained when 1.2×10^{-5} M TPN is added without ATP. Such effects of ATP without added TPN were never obtained in the case of the plant extracts.

Quantitative Assay for TPN Malic-Enzyme

Attempts were made to determine a range of enzyme concentration in which the rate of O_2 consumption would be directly proportional to the quantity of enzyme added. As would be expected from the foregoing results, no adequate proportionality was observed unless the destruction of TPN was prevented. Fig. 5. shows the lack of proportionality obtained with 22 γ TPN per vessel, but no other added nucleotide. This experiment illustrates particularly the fact that there

is a range of protein concentration in which an increase in amount of protein actually causes a decrease in the quantity of O_2 consumed over a given period of time. The disadvantage of using such a procedure for quantitative assay is obvious. When, however, the destruction of TPN is prevented by addition of adequate amounts of ATP, linear

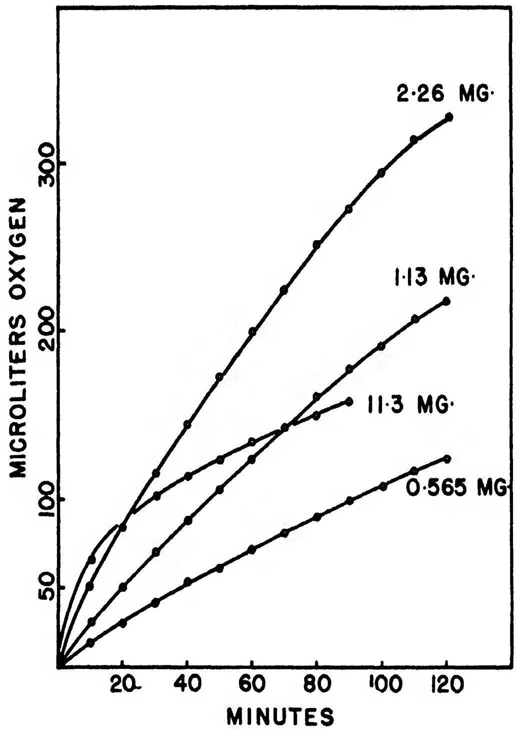

FIG. 5. Oxygen uptake with different amounts of wheat germ. All vessels contained 0.0625 *M* *l*-malate; 0.05 *M* glycylglycine buffer, pH 7.4; 0.0025 *M* $MnCl_2$; 0.0025 *M* KCN (neutralized to pH 7.4); 20 mg. yellow enzyme; 7×10^{-6} *M* TPN; water to a volume of 4.0 ml., and the indicated amounts of wheat germ enzyme, Type C. Reaction started by tipping in yellow enzyme and TPN from the side arm after temperature equilibration. Gas, O_2; temperature 37°C. KOH in center well.

proportionality was observed over a wide range of protein concentration as shown in Fig. 6. The different curves do not pass through the origin because there is a small and reproducible consumption of O_2 when no wheat germ protein is present. The blank in this experiment is due to the yellow enzyme since omission of the latter gives no measureable O_2

uptake whatever. Some crude plant preparations also gave a small O_2 consumption without added yellow enzyme. Appropriate corrections for these blanks can readily be made for each preparation used.

The assay system described suffers from the disadvantage that the behavior of the peroxide formed varies in the different plant prepara-

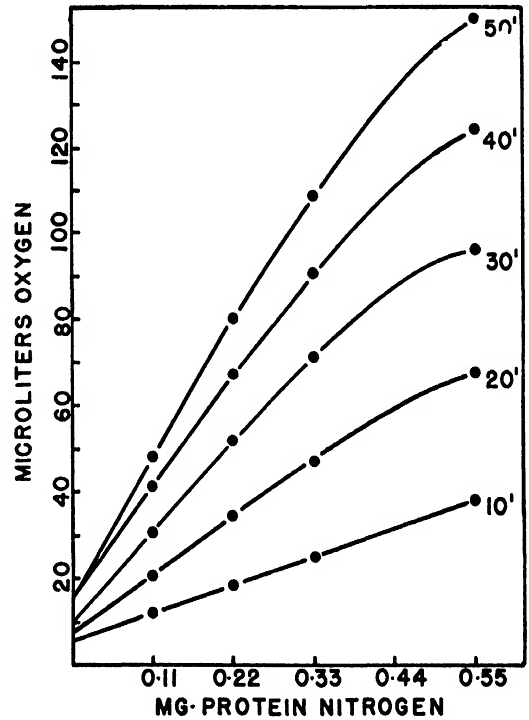

FIG. 6. Oxygen uptake as a function of wheat germ concentration. All vessels contained 0.083 *M* *l*-malate; 0.067 M glycylglycine buffer, pH 7.4; 0.003 M $MnCl_2$; 0.0015 *M* ATP; 1.7×10^{-5} *M* TPN; 20 mg. yellow enzyme; water to a volume of 4.0 ml., and the amounts indicated of wheat germ enzyme, Type B, expressed as protein nitrogen. Curves are O_2 uptake for periods of 10, 20, 30, 40, and 50 min. after tipping in yellow enzyme, TPN, and ATP from the side arm after temperature equilibration. Gas. O_2; temperature, 37°C. KOH in center well.

tions. It is, therefore, necessary in each case to examine the balance between malate disappearance or pyruvate formation and O_2 consumption in order to interpret correctly the quantitative results with respect to malic enzyme content.

Distribution of TPN Malic-Enzyme

The manometric test method for TPN malic-enzyme was applied to 6 plant preparations other than wheat germ. In each case, tests were run with and without ATP and DPN in order to ascertain whether these substances always had the same effect. Blanks omitting malate always showed negligible O_2 uptake. Some preparations gave results qualitatively similar to those observed with wheat germ. Among these were the samples prepared from beets, carrots, spinach, and peas. In each case, approximately the same number of carboxylase units were employed per test as was the case with wheat germ.

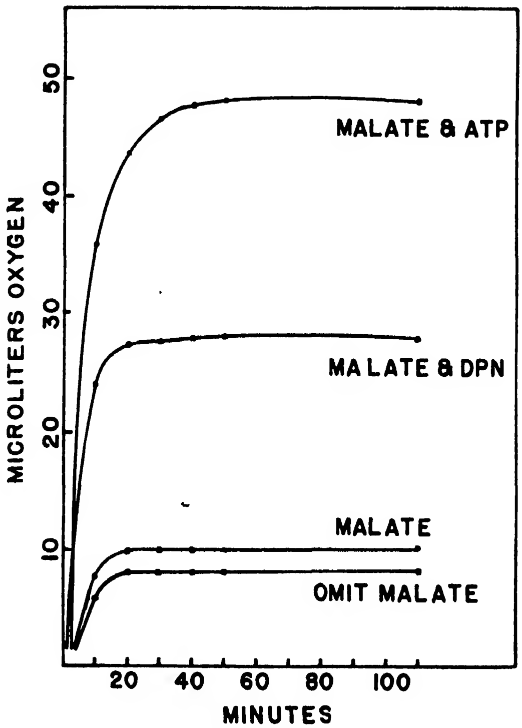

FIG. 7. Demonstration of TPN-malic enzyme in parsley root. All vessels contained 0.05 *M* glycylglycine buffer, pH 7.4; 0.0025 *M* $MnCl_2$; 20 mg. yellow enzyme; 1×10^{-5} *M* TPN; 12 mg. parsley root; 0.0025 *M* KCN (neutralized to pH 7.4); and water to total volume of 4.0 ml. Additions as indicated were: 0.0625 *M* *l*-malate; 0.0013 *M* ATP; and 2×10^{-4} *M* DPN. Reaction started by tipping in TPN and yellow enzyme together with ATP or DPN when indicated from side arm after temperature equilibration. Gas, O_2; temperature, 37°C. KOH in center well.

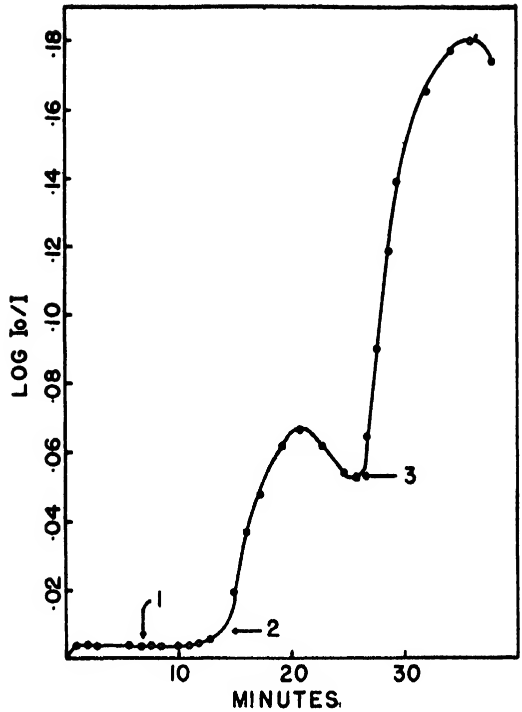

FIG. 8. Demonstration of TPN-malic enzyme with cytochrome reductase. Reduction of cytochrome c determined spectrophotometrically at 550 mμ. Corex cell, d = 1.0 cm. Temperature, 25°C. Measurements were made against a blank which received all additions except wheat germ enzyme. Optical density corrected for changes on dilutions. 0.2 ml. 0.5 M glycylglycine buffer, pH 7.4; 0.15 μM cytochrome c; 0.112 mg. cytochrome c reductase; 6.9 mg. wheat germ, Type B; 0.3 ml. 0.5 M l-malate; 0.1 ml. 0.01 M $MnCl_2$; and water made to a volume of 3.3 ml. At 0 time, 1.4 γ TPN added. At 1, 321 γ DPN added. At 2, 1.4 γ TPN added. At 3, 1.4 γ TPN added. At 40 min. (not shown on graph) addition of 14 γ TPN caused a change in log I_0/I of 0.394.

Parsley root and parsnip preparations tested likewise gave similar results, except that virtually no O_2 consumption was observed with malate and TPN unless either ATP or DPN was added. A typical experiment with parsley root is shown in Fig. 7. It may be noted that the enzyme employed in this epxeriment was one which had previously given negative results when tested for the malic enzyme by the direct spectrophotometric procedure. It is obvious that the malic activity is actually present, but that the preparation apparently contains a high

concentration of TPN-ase. Results of this type illustrate most clearly the questionable nature of negative results for the malic enzyme. Even in the presence of ATP, the use of an excess of plant protein may give virtually negative results in the manometric test system. For example, when the enzyme concentration was increased 5-fold in the experiment of Fig. 7, no significant uptake of oxygen was observed. In these experiments no attempt was made to correlate quantitatively the malic and carboxylase activities in the various samples, nor for that matter has there been any effort to compare TPN-ase activities of the different preparations.

Coupling of TPN Malic-Enzyme with Cytochorme c Reductase

TPN malic enzyme may also be detected spectrophotometrically by following reduction of cytochrome c at 550 mμ in the presence of cytochrome c reductase (25, 26). This method is extremely sensitive and requires only very small quantities of TPN. However, because of this latter fact, it is particularly susceptible to the TPN-destroying enzymes in the plant preparations. Where such factors are not present, reduction is readily observed. Where the TPN-ases are present, it is possible to overcome their effect by the addition of other nucleotides such as DPN. Experiments of this type were run with wheat germ enzyme and with parsnip. The results with the wheat germ enzyme are shown in Fig. 8. When 1.4 γ TPN were added at zero time, no reduction of cytochrome c occurred. At time 1, 321 γ DPN were added, with no change in light absorption. However, when an additional 1.4 γ TPN were added at time 2, reduction was observed. After 7 min. an apparent reoxidation of cytochrome c[8] seemed to occur. At time 3 another 1.4 γ TPN were added and reduction again occurred. Muscle adenylic acid gave results similar to DPN.

All these results can be interpreted in a manner similar to that given in the section on manometric experiments, *i.e.*, the enzyme preparations contain a TPN-ase the action of which can be prevented by DPN, ATP, and adenylic acid.

[8] The nature of this reoxidation has not been examined. It may be too rapid to be accounted for entirely as an autoxidation by air. This point requires additional investigation.

Discussion

The work reported in this paper was designed to determine whether a TPN-specific enzyme catalyzing the reversible oxidative decarboxylation of malic acid is present in a variety of OAA carboxylase preparations available from various plant sources. A need was felt for a method which could be widely applied to a group of preparations and, if possible, could be used for a quantitative assay. The manometric procedure described for detection of the malic activity can be applied in this fashion provided care is taken to avoid the destruction of TPN by addition of muscle adenylic acid, ATP or DPN. Even in the presence of added nucleotides, an excess of enzyme must be avoided. Care must also be taken, in a quantitative application of the method, to determine the ratio of malate oxidation to O_2 utilization in order to interpret correctly the O_2 uptake in terms of malic enzyme activity.

The presence of TPN malic enzyme in the 7 plants studied shows that the distribution of the enzyme is extensive. No plant β-carboxylase preparation tested to date has been found which does not contain the malic-enzyme, provided precautions are taken to prevent TPN destruction. The successful demonstration of the activity therefore indicates a close relationship between the dehydrogenation and the decarboxytion such as Ochoa, Mehler and Kornberg (2) have found to be the case in pigeon liver. However, insufficient data are at hand to warrant the final conclusion that the two activities are associated with one protein.

The widespread distribution of the malic enzyme indicates that a large number of cell-free plant preparations are capable of causing the synthesis of malate from pyruvate and CO_2, provided a source of reduced TPN is available. Whether radiant energy may effect a reduction of TPN through the mediation of chlorophyll is a question which requires further experimentation. The dependence of some "dark reactions" on TPN has been demonstrated. It is not impossible that such earlier results as those of Frenkel (27) may be explained in these terms. Frenkel found that dark fixation of CO_2 in cell sap of *Nitella* was dependent on intact cell structure. If the TPN were destroyed on disintegration of the cells, such a result might be expected, even though the proteins capable of catalyzing the reductive carboxylation of pyruvate to malate were present.

Although the TPN-destroying factors encountered in this work were

largely obstacles to the demonstration of the malic enzyme, their widespread distribution is a fact of interest in itself, and suggests that great care must be exercised in interpreting stimulatory effects of such substances as adenylic acid on an oxidation system dependent on the pyridine nucleotides. The experiments made were not designed to determine the mode of action of the TPN-ase or the method whereby adenylic acid, ATP or DPN exert their effects. The disappearance of the 340 mμ band of reduced TPN might indicate a splitting of the nicotinamide-ribose bond. However, nicotinamide was not effective in preventing destruction, as it was found to do in the case of DPN-nucleosidase described by Mann and Quastel (21) and by Kornberg and Lindberg (22). On the other hand, the fact that substances having in common the structure of adenylic acid can prevent the destruction of TPN suggests that inactivation may involve hydrolysis near the adenylic acid portion of the molecule. The protective action of ATP and DPN may also depend on hydrolysis of these compounds to adenylic acid, which may be the only active agent. Kornberg (28) has described a pyrophosphatase which splits ATP, DPN, TPN, and flavine-adenine nucleotide. He has observed a protective action of DPN on the other 3 compounds and this suggests a similarity to the effects reported in this paper. However, the effect of adenylic acid was not reported by Kornberg.

The possibility cannot be excluded that more than one factor are involved in the destruction of TPN by the crude plant preparations employed. Additional data are required before any conclusions can be drawn as to the identity or lack of identity of this destructive factor with those enzymes, which have been described by other authors.

Acknowledgments

We are grateful to Dr. E. A. Evans, Jr., for his helpful advice and generous permission to use his facilities. We are also indebted to Dr. T. R. Hogness for permission to use facilities made possible by a grant from the Rockefeller Foundation.

Summary

The widespread distribution of factors which destroy TPN has been found to constitute the main difficulty in demonstrating the presence of a TPN-specific malic enzyme in plant tissues. The action of the destroying factors can be prevented by ATP, DPN, and adenylic acid. These facts have been applied to devise a generally applicable mano-

metric assay procedure for the detection and the quantitative estimation of the malic enzyme.

The assay procedure has been used to study the distribution of the enzyme. Seven different plant sources (wheat germ, beets, spinach, carrots, parsley root, parsnip, and peas) were all found to contain the malic enzyme. All of these preparations also contained oxalacetic carboxylase.

The results suggest a possible close association between the malic activity and the carboxylase, and indicate, further, that soluble enzymes which can cause the reductive carboxylation of pyruvate to malate are widely distributed in the tissues (roots, leaves and seeds) of higher plants.

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The Genesis of Auxin during the Decomposition of Proteins

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Introduction

The term "auxin protein" has been applied to a protein isolated from spinach leaves, which upon alkaline hydrolysis yielded about 0.02–0.09 γ of auxin, calculated as indoleacetic acid/mg. of the protein (1, 8). As shown below, the auxin appears indeed to be indoleacetic acid but amounts to far less than one molecule per molecule of protein (mol. wt. *ca.* 2×10^5). In no case was the indoleacetic acid obtained without hydrolyzing the protein. On the contrary, in order to produce the auxin, the protein was subjected to treatments of many hours in 0.1 *N* NaOH at 100°C., a procedure which has been shown to give comparable quantities of indoleacetic acid from a variety of proteins not in any way related to plants and not having any enzymatic properties. Despite the small quantities of auxin obtained, the drastic treatment of the protein required to obtain them, and the non-specificity of the reaction, Wildman and Bonner (8) suggest that "a reasonable working hypothesis may be that the auxin of the protein is related in some way to the specific enzymatic activity of the material," and that "probably the bound auxin of Fraction I [the auxin protein] should not be regarded as a precursor or storage form of auxin, but rather as the biochemically active form of the growth substance."

The following experiments were carried out to test this hypothesis and, in general, to provide a background for the appraisal of any possible physiological or chemical significance attributable to the appearance of a minute quantity of indoleacetic acid in the hydrolyzate of a protein.

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To determine the specificity of the reaction giving rise to the indoleacetic acid, a group of proteins from widely varying sources was subjected to the treatment found optimum for obtaining indoleacetic acid from the spinach "auxin protein." This investigation reveals that the property of yielding small quantities of indoleacetic acid upon alkaline hydrolysis is by no means specific to any one enzyme, or to any one protein, nor is it specific to proteins of botanical origin, but, on the contrary, seems to be a widespread property of tryptophan-containing proteins of whatever origin or function.

Materials and Methods

The quantities of auxin occurring in the hydrolyzates of the proteins were determined by means of the standard *Avena* test as described by Went and Thimann (7). Each test was standardized by including a sample consisting of a solution of indoleacetic acid of known concentration. The curvatures produced by the samples being tested were compared with the curvatures produced by the known concentrations of indoleacetic acid, and the activities of the samples are expressed in mg.-equivalents of indoleacetic acid. The assumption that the *Avena* curvatures produced by the extracts of the protein hydrolyzates are due to indoleacetic acid rather than some other auxin is based on the observations that the active compound is an acid, that it is relatively stable to boiling 0.1 *N* NaOH, and particularly, that it gives a positive test in the modified Salkowski reaction described by Tang and Bonner (6) as specific to indoleacetic acid.

Efforts were made to obtain indoleacetic acid or any other auxin from the proteins particularly the spinach Fraction I, by treatments which should leave the protein intact. Among the methods tried were prolonged dialysis against distilled water and various buffers, precipitation at the isoelectric point, washes with acids, bases, and organic solvents. None of these was successful, but rather confirmed the conclusion that hydrolysis of the protein is a necessary condition for obtaining any auxin from it. The results here reported are those obtained by hydrolyzing the proteins in dilute NaOH solutions, a procedure also used by Wildman and Bonner (8).

To assay the auxin in such a hydrolyzate by the *Avena* method it is brought to pH 3 by the addition of HCl, and is extracted with ether. The extract is concentrated by boiling off the ether until a volume of about 1 ml. remains. This 1 ml. is then quantitatively transferred into a vial containing 0.8 ml. of hot agar, whereupon the ether evaporates and leaves the dissolved material in the agar. The agar, which now contains any auxin which may have been present in the protein hydrolyzate, is serially diluted so that at least one concentration will be of the proper magnitude for the assay. Each dilution is then poured into a mold and cut into 12 equal-sized blocks which are used in the *Avena* test as described above.

The proteins investigated were the following:

Spinach Cytoplasmic Protein (*Fraction I*). This is the "auxin protein" of Wildman and Bonner (8), the preparation of which they have described. The samples used were generously supplied by Dr. Wildman or prepared under his supervision.

Ovalbumin. A thrice-recrystallized sample prepared by the method of Keckwick and Cannon (4) and supplied through the courtesy of Dr. George Feigen.

β-Lactoglobulin. A twice-recrystallized sample prepared by the method of Palmer (5) and obtained through the courtesy of Dr. John Cushing.

Chymotrypsin. A crystalline preparation obtained from the Plaut Research Laboratory, Lehn and Fink Products Corp., Bloomfield, N. J.

Fibrin. "Difco, Desiccated" obtained from Difco Laboratories, Inc., Detroit, Mich.

Serum Albumin. A crystalline sample obtained from Professor Norman Horowitz.

Experimental

The optimum conditions for obtaining auxin from proteins upon alkaline hydrolysis were investigated, using the spinach Fraction I. In all of these experiments the reaction mixtures were 5 ml. in volume, and the normality of base specified refers to the final concentration in this volume. The reactions were carried out in 30 ml. test tubes equipped with air condensers, and heated in a boiling water bath. From Fig. 1,

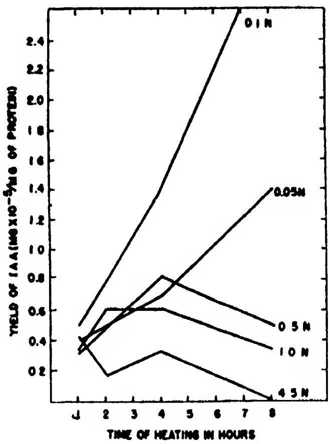

Fig. 1. Auxin yields, calculated as indoleacetic acid equivalents, obtained from spinach Fraction I, the "auxin-protein," upon refluxing at 100°C. with NaOH of various concentrations.

showing the yields obtained with various concentrations of NaOH, it is evident that 0.1 *N* is the most favorable concentration of alkali for obtaining auxin from the spinach protein, when the hydrolysis is carried out at 100°C.

These conditions were then applied to other proteins. A comparison of the rates of appearance and maximum yields of auxin in the hydrolyzates of various proteins is shown in Fig. 2. Naturally, not all proteins could be expected to react in the same way to a given set of conditions. The experiment shows, however, that the reaction giving rise to the auxin is by no means unique to the spinach Fraction I but is displayed by many other proteins when they are similarly treated. Positive *Avena* tests were also obtained when the protein of *Avena* coleoptiles, trypsin, and zein (a sample of un-

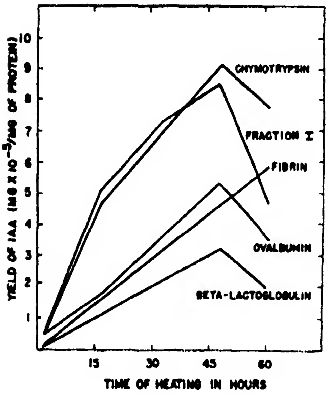

FIG. 2. Auxin yields, calculated as indoleacetic acid equivalents, obtained from several proteins upon refluxing at 100°C. with 0.1 N NaOH.

known purity) were treated, although the investigation of these proteins was not extensive enough to determine their yields with certainty. Gelatin, which contains no tryptophan, and pepsin yielded no auxin.

Since a plausible explanation for the appearance of trace amounts of auxin in the hydrolyzates of the proteins studied seemed to be that they arise through the oxidative deamination of tryptophan to indoleacetic acid, the abundance of this amino acid in the proteins was compared with their yields of auxin. For this purpose the tryptophan content of the spinach Fraction I was determined by the Adamkiewicz-Hopkins method (2), while values for the other proteins were taken from the literature. In Table I are listed the tryptophan contents and auxin yields of the various proteins.

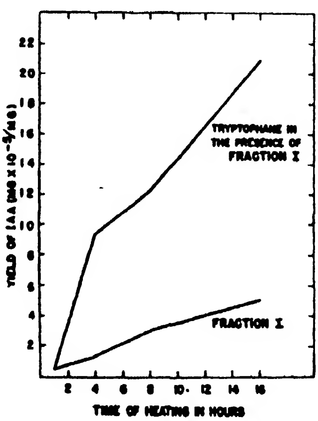

FIG. 3. Auxin yields, calculated as indoleacetic acid equivalents, obtained upon refluxing Fraction I at 100°C. in 0.1 N NaOH, with and without added tryptophan (1 mg.).

TABLE I

Tryptophan Content and Auxin Yields of Various Proteins

Protein	Tryptophan *per cent*	Maximum IAA equivalents obtained on heating in 0.1 *N* NaOH at 100°C.
Chymotrypsin	5.5	9.2×10^{-2} γ/mg. protein
Fraction I	4.1	8.5
Fibrin	3.0	5.8
β-Lactoglobulin	1.9	3.2
Ovalbumin	1.3	5.4
Serum albumin	0.2	0.1
Gelatin	0.0	0 (<0.0002)

Further to test the hypothesis that it is tryptophan which gives rise to the auxin in the protein hydrolyzates, tryptophan was subjected to the conditions of the protein hydrolysis. The results of these experiments, presented in Fig. 3, show that tryptophan is indeed converted to some extent to indoleacetic acid under the conditions used to obtain auxin from the proteins.

Discussion

Inasmuch as the capacity to yield small amounts of auxin proves to be commonplace among tryptophan-containing proteins, and in fact appears to be roughly proportional to the tryptophan contents of the proteins, it is interesting to consider whether this auxin could be indoleacetic acid originating from the tryptophan. Significant in this consideration is the fact that, under the conditions of the hydrolysis used to obtain indoleacetic acid from the proteins, tryptophan is released into a warm alkaline reaction medium. It is known that, under these conditions, tryptophan undergoes oxidative deamination to indoleacetic acid (3). It is not surprising, therefore, that tryptophan treated along with the protein in 0.1 N NaOH at 100°C. contributes to the yield of auxin acid. The results of such an experiment as presented in Fig. 3 are, however, difficult to interpret quantitatively for the following reason. The free tryptophan is exposed to the warm alkali from the beginning of the experiment. Whatever indoleacetic acid is produced appears early and is then exposed to the destructive action of the reaction medium. (Note how the yields fall off with time in Fig. 2.) The tryptophan from the protein, on the other hand, is only slowly released into the medium and, therefore, whatever indoleacetic acid is formed from it appears later. The indoleacetic acid from the added tryptophan is

exposed to the destructive action of the reaction medium for a longer period of time than the indoleacetic acid from the peptide tryptophan. The yields cannot, therefore, be compared directly, and, moreover, if suitably small quantities of tryptophan are added, and long periods of digestion used, then the effect of the added tryptophan can be lost entirely.

That the auxin found in the hydrolyzates of the proteins investigated arises through the oxidative deamination of tryptophan is offered as an hypothesis consistent with the observations. The fact remains, however, that, whatever the source of the auxin may be, the same treatment produces comaprable quantities in the hydrolyzates of such widely varying proteins as chymotrypsin, fibrin, β-lactoglobulin, ovalbumin and serum albumin, in addition to the spinach Fraction I. This fact makes it appear unwarranted to attribute any physiological significance to the auxin obtainable from the spinach protein which cannot also be assigned to the auxin obtainable from the other proteins. This type of so-called "bound auxin" would, therefore, appear to be associated neither with any special enzymatic activity of the protein, nor specifically with plant growth or plants.

Acknowledgments

Thanks are due Miss Martha Kent for her assistance in many of the experiments reported, and to Professor James Bonner and Dr. Sam Wildman for their cooperation.

Summary

1. The treatment found to give optimum yields of auxin from spinach Fraction I, the "auxin protein," was refluxing at 100°C. in 0.1 *N* NaOH.

2. Chymotrypsin, fibrin, β-lactoglobulin, ovalbumin, serum albumin and other proteins all yielded an auxin believed to be indoleacetic acid when subjected to this treatment.

3. The yields of auxin from the protein hydrolyzates were roughly proportional to the tryptophan contents of the proteins.

4. When tryptophan was added to the reaction medium, the yield of indoleacetic acid was increased.

5. It is suggested that the auxin from the proteins arises through oxidative deamination of tyrptophan to indoleacetic acid in the hot alkaline medium in which the proteins are hydrolyzed.

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Antiscorbutic Substances. 3-Methyl-L-Ascorbic Acid and 1-Methylheteroascorbic Acid

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Introduction

It is generally accepted that, for an analog of ascorbic acid to have antiscorbutic activity, it is essential that, among other things, all the hydroxyl groups in the molecule must be free.

However, 3-methyl-L-ascorbic acid appears to show, on the basis of relatively non-specific preventive and curative methods, with growth only as the criterion (1,2) as much as 1/10 of the activity of L-ascorbic acid. The responses to the elevated doses of the compound are, however, not typical of those observed with ascorbic acid, and it was suggested that preliminary *in vivo* conversion to ascorbic acid is involved.

Previous investigations (3,4,5) have established that a critical ascorbic acid intake (0.225 mg./day) is necessary for normal osteoblastic function in the guinea pig and the "alkaline" phosphatase level of the blood has been shown to be the most sensitive and earliest index of this function. The restoration of normal serum phosphatase levels upon the administration of a critical dose of ascorbic acid to scorbutic animals is strikingly prompt (Figs. 1 and 2) and can be used as a measure of antiscorbutic activity (3). Responses that occur only after prolonged administration would be suggestive of the necessity for preliminary conversion of the compound to ascorbic acid.

Using the phosphatase bioassay, studies were made to determine (1) the exact antiscorbutic potency of 3-methyl-L-ascorbic acid, and (2) whether preliminary conversion to L-ascorbic acid appears to be involved; and using the classical histological technique, to determine unequivocally whether the compound shows (1) true antiscorbutic properties, and (2) whether it may act competitively with L-ascorbic acid.

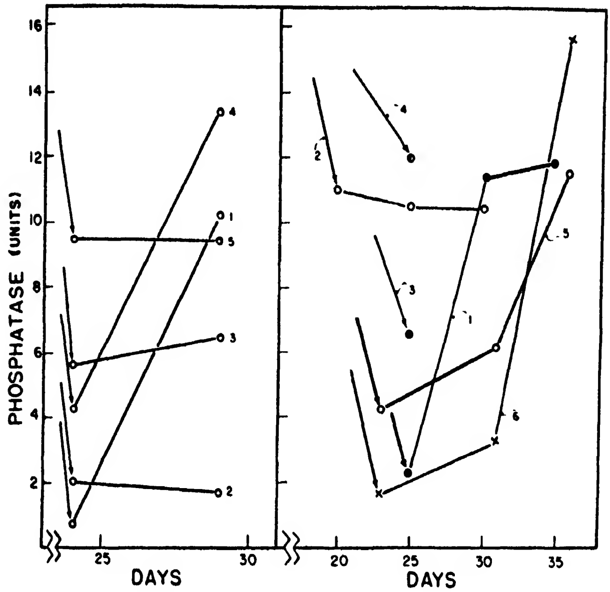

FIG. 1 FIG. 2

FIG. 1. Serum phosphatase responses of animals maintained on a scorbutogenic diet for 24 days, then fed various supplements for 5 days.

Curve 1, animals fed 0.225 mg. ascorbic acid; Curve 2, 6.75 mg. 3-methyl-L-ascorbic acid; Curve 3, 11.25 mg. 3-methyl-L-ascorbic acid; Curve 4, 1 mg. 1-methylheteroascorbic acid; and Curve 5, fed 0.225 mg. ascorbic acid throughout the preparative and test periods.

FIG. 2. Typical serum phosphatase responses of animals fed ascorbic acid, 1-methylheteroascorbic acid and 3-methyl-L-ascorbic acid.

Curve 1, animals fed a scorbutogenic diet for 25 days then supplemented by 0.225 mg. ascorbic acid for 10 days; Curve 2, fed the diet supplemented by 0.25 mg. ascorbic acid throughout; Curve 3, fed the diet supplemented by 11.25 mg. 3-methyl-L-ascorbic acid for 25 days; Curve 4, fed the diet supplemented by 1 mg. 1-methylheteroascorbic acid; Curve 5, fed the diet supplemented by 5.6 mg. 3-methyl-L-ascorbic acid for 23 days followed by 11.25 mg. (intraperitoneally) for 13 days; and Curve 6, supplemented by 2.25 mg. 3-methyl-L-ascorbic acid for 23 days followed by 11.25 mg. (intraperitoneally) for 13 days.

EXPERIMENTAL METHODS

The methods for the maintenance and treatment of the animals[1] as well as the estimation of phosphatase are those described in previous studies (3,4,5,7). Test animals invariably showed evidence of scurvy and characteristically low serum phosphatase levels in 18–25 days. Control animals responded characteristically to the

[1] We are extremely grateful to Mr. Leonard Stutman for considerable assistance in the care and preparation of the animals.

critical level of 0.225 mg. ascorbic acid daily after 5 days administration and, at levels below this, there was no phosphatase response.

Histological Methods

The midsagittal section of the head of each animal was cut in transverse sections at predetermined points at different levels of the incisor teeth. The decalcified (5% HNO_3) sections were cut, mounted and stained with hematoxylin and eosin. Some sections were also stained by the Wilder's silver-stain method.

Synthesis of 3-Methyl-L-Ascorbic Acid and of 1-Methylheteroacsorbic Acid.

In addition to one sample of 3-methyl-L-ascorbic acid kindly supplied by Dr. C. S. Vestling and another by Dr. M. C. Rebstock, to whom we express our sincere thanks, both 3-methyl-L-ascorbic acid and 1-methylheteroascorbic acid were prepared by a slight modification of the method used by Vestling and Rebstock (2). The preparation showed no depression of the melting point when admixed with the sample obtained from Dr. Vestling (m.p. 124–125°C.) and contained no measurable amount of ascorbic acid, as indicated by a completely blank titration with 2,6-dichlorophenol indophenol reagent.

The 1-methylheteroascorbic acid obtained was recrystallized from hot nitromethane and yielded a crop of orange blocks which melted at 159–161°C. (with decomposition). Haworth's preparation (6) melted at 162°C.

Results

3-Methyl-L-Ascorbic Acid

From the results shown in Fig. 2 it is evident that 3-methyl-L-ascorbic acid fed to the extent of 30 times the critical dose of L-ascorbic acid showed no protective action. On the other hand, when 50 times the critical dose was fed, most of the animals were protected, as shown by histological examination as well as by the phosphatase level of the serum, indicating that the compound is 1/50th as active as L-ascorbic acid. However, the results of curative tests show a striking difference from the response anticipated if 3-methyl-L-ascorbic acid were merely 1/50th as active as L-ascorbic acid. The histological and phosphatase responses obtained upon the administration of various analogs of ascorbic acid (5), while quantitatively different, are essentially the same when the quantities administered are adjusted to be equivalent to the desired amount of ascorbic acid, in that the effects are evident in as short a time as 24 hr. and invariably, in less than 5 days (Fig. 2). The results obtained here indicate a markedly delayed response, both in

serum phosphatase elevation and in histological tooth structure repair, when 3-methyl-L-ascorbic acid is administered. Even when 50 times the critical dose of L-ascorbic acid is administered intraperitoneally, there was no significant response after 5 or even 8 days, but, if the compound is administered over a prolonged period of 12–13 days, the response is apparent (Fig. 2). It would appear, therefore, that preliminary conversion of the methylated compound to L-ascorbic acid is essential before activity is manifested.

1-Methylheteroascorbic Acid

Using doses of 1 mg. per day (about 4 times the critical dose of L-ascorbic acid), preventive and curative experiments indicate that the

TABLE I

Histological Examination of Some Typical Animals Fed Ascorbic Acid, 3-Methyl-L-Ascorbic Acid, 1-Methylheteroascorbic Acid, or Mixtures of the Compounds

Animal	Treatment[a]	Phosphatase response	Histological response[b]
24	0.5 mg. A. A. for 23 d.	Phosphatase, 9.5 units	No indication of scorbutic lesions
10	2.25 mg. MAA for 23 d.	Phosphatase fell to 3.8 units	Very scorbutic
11	2.25 mg. MAA for 23 d., then 11.25 mg. for 13 d.	Phosphatase at 23 d. fell to 1.7 units, then rose to final value of 16.5	Scorbutic, followed by very mild repair, then very rapid repair
14	5.6 mg. MAA for 23 d.	Phosphatase fell to 2.6 units	Scorbutic, complete cessation of dentine formation
15	Same as No. 14, then given 11.25 mg. for 12 days	Phosphatase fell to 4.2 units at 23 d., then rose after 12 d. to 11.5 units	Scorbutic, followed by very mild repair, then very rapid repair
18	11.25 mg. MAA for 26 d.	Phosphatase, 12.5 units	Subacute scorbutic condition still laying down irregular dentine

TABLE I (*Continued*)

Animal	Treatment[a]	Phosphatase response	Histological response[b]
19	11.25 mg. MAA for 26 d.	Phosphatase, 9.5 units	Mild scorbutic condition
20	11.25 mg. MAA for 23 d.	Phosphatase, 5.5 units	Mild scorbutic condition
21	11.25 mg. MAA for 23 d.	Phosphatase, 6.1 units	Mild scorbutic condition no repair
32	Scorbutogenic diet for 23 d., then 11.25 mg. MAA for 5 d.	Phosphatase at 23 d., 5.8 units; at 28 d., 6.4 units	Very scorbutic, no significant repair
30	Scorbutogenic diet for 23 d., then 1 mg. HAA for 5 d.	Phosphatase fell to 2.2 units at 23 d., then rose to 7.8 units at 28 d.	Markedly scorbutic followed by good repair
22	11.25 mg. MAA plus 0.5 mg AA for 25 days	Phosphatase, 8.5 units	No indication of scorbutic condition. Same as animal 24
9	Scorbutogenic diet for 18 d., the 0.25 mg. AA for 5 days	Phosphatase fell to 3.6 units at 18 d., then rose to 10.1 units at 23 days	Very scorbutic, then mild repair

[a] AA = L-ascorbic acid.
MAA = 3-Methyl-L-ascorbic acid.
HAA = 1-Methylheteroascorbic acid.
[b] A detailed histological description will be reported separately.

response is like that of L-ascorbic acid and unlike that of 3-methyl-L-ascorbic acid, in that there is no delay in the manifestation of its antiscorbutic activity (Fig. 2), suggesting rapid demethylation to active L-ascorbic acid.

Competitive Action between 3-Methylascorbic Acid and L-Ascorbic acid

Experiments were carried out in which animals were fed 11.25 mg. of 3-methyl-L-ascorbic acid and 0.5 mg. of L-ascorbic acid. There was no indication of an antagonistic effect between the two compounds (Table I) under these conditions.

Summary and Conclusions

1. Bioassays employing (1) the serum phosphatase response and (2) the histological tooth structure examination indicate that 3-methyl-L-ascorbic acid is only 1/50th as active antiscorbutically as L-ascorbic acid. 1-Methylheteraoscorbic acid shows strong antiscorbutic activity.

2. Both the blood phosphatase and histological responses to the administration of large doses of 3-methyl-L-ascorbic acid are markedly delayed in contrast to the prompt responses observed with L-ascorbic acid; suggesting that preliminary conversion of 3-methyl-L-ascorbic acid to L-ascorbic acid probably occurs.

3. There appears to be no biochemical antagonism between 3-methyl-L-ascorbic acid and L-ascorbic acid when mixtures of the two are fed, the former in relatively large and the latter in relatively small amounts.

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Chemical Composition of Normal Bone Marrow

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Introduction

In a previous paper, correlations were shown between the water, lipide, residue, total nitrogen, and lipide nitrogen components of normal rabbit marrow (1). In the present paper, similar relationships are shown for the marrow of other animals. In addition, a study was made of the sulfur, nitrogen, and phosphorus distribution in marrow. A number of isolated results may be found in the literature. Singher and Marinelli (2) stated that rat marrow had a high sulfur content, but they gave no values. A value of 25–30 mg.-% of acid-soluble phosphorus was recorded by Lutwak-Mann (3) for rabbit marrow. The nitrogen components of normal and anemic rat marrow were determined by McCoy and Schultze (4). An attempt is made, in this paper, to obtain a broader picture of the composition of bone marrow, so that deviations from normal can be related to alterations in hemopoietic function.

Experimental

Bone marrow was obtained from rabbits, rats, and guinea pigs of all ages, from a 4 month old cat, a 2 month old beef calf, a frog (*Rana pipiens*), hogs, bulls, and dogs. The cat and dogs were killed by nembutal anesthesia, and the other animals by exsanguination following a blow at the base of the skull. With the exception of the hog, and a few samples of beef marrow obtained at a slaughter house, all marrow samples were removed immediately after death. The method of analysis for the gross components, in marrow taken from different bones, was the same as previously described (1), with the exception that the fat was extracted once with petroleum ether as well as 3–4 times with alcohol-ether (3:1). For rabbit marrow the petroleum ether was not necessary, but for some of the other samples, especially beef, the bulk of the fat did not dissolve readily in the alcohol-ether mixture. For the smaller animals, the marrow from 2 or more bones had to be grouped to obtain adequate samples. The data were subjected to correlation analysis, as previously described (1). The symbols W, L, R, N, and N_L are again used to represent percentage concentrations of the

TABLE I
Composition of Femur Marrow

Animal	Age months	Water	Lipide	Residue	Nitrogen	Lipide nitrogen per cent of lipide
		per cent	*per cent*	*per cent*	*per cent*	
Guinea pig	0.8	70.4	12.1	17.5	2.54	0.87
Guinea pig	24.	70.8	10.4	18.8	2.73	1.38
Rat	1.8	73.3	5.6	21.1	3.06	2.13
Rat	26.	67.7	14.0	18.3	2.75	0.81
Cat	4.	50.1	38.2	11.7	1.84	0.33
Beef	1.5	41.3	47.7	11.0	1.51	0.18
Beef	>50.	9.2	88.2	2.6	0.23	0.03
Hog	>12.	8.1	90.1	1.8	0.20	0.03
Rabbit	1.2	73.9	12.5	13.6	2.09	0.57
Rabbit	24.	36.0	55.7	8.3	1.21	0.09
Dog	>24.	20.8	72.1	7.1	0.87	0.10
Frog	18.	65.4	28.4	6.2	0.87	0.11

water, lipide, residue (lipide-free solids), and nitrogen in the whole bone marrow, and lipide nitrogen as per cent of the lipide, respectively.

In addition to the gross analyses, protein-free filtrates of certain marrows were prepared. The marrow was ground in a mortar to give a homogeneous mixture and an aliquot of 2–2.5 g. was weighed into a 25 ml. volumetric flask. Ten ml. of water were added and the mixture allowed to stand at room temperature for 1 hr. to hemolyze the cells. At this time, 12.5 ml. of 20% trichloroacetic acid were added and made up to volume with water. The protein-free filtrate was obtained and analyzed for non-protein nitrogen by the Kjeldahl method, inorganic phosphate by the method of Tisdall (5) as modified by Kaucher *et al.* (6), and the distribution of sulfur. The method used

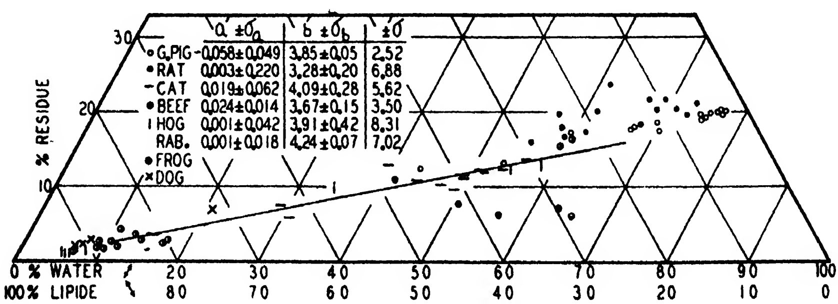

FIG. 1. Composition of normal bone marrow plotted on triangular coordinates. The values given in the insert are for the equation, $W = aL + bR$, with the standard error of estimate of the equation. The line represents the composition of marrow of rabbits 1–24 months of age (1).

for the distribution of sulfur was essentially that of Marenzi *et al.* (7), removing the phosphate with zirconium oxychloride at a pH of 9.0 ± 0.2 and isolating the benzidine sulfate. The sulfate, however, was titrated by the method of Cope (8). The accuracy of this method was the same as found by Power and Wakefield (9). The total sulfur and phosphorus contents of the marrow were determined on another aliquot of 0.1–0.4 g. A micro modification of the HNO_3 and $HClO_4$ oxidation of Evans and St. John (10) was used, and sulfate and phosphate determined as described above. About 95% of the sulfur in 5 mg. of methionine was recovered following oxidation with 3.5 ml. HNO_3 and 1 ml. $HClO_4$. A third aliquot was analyzed for the major components as previously described (1).

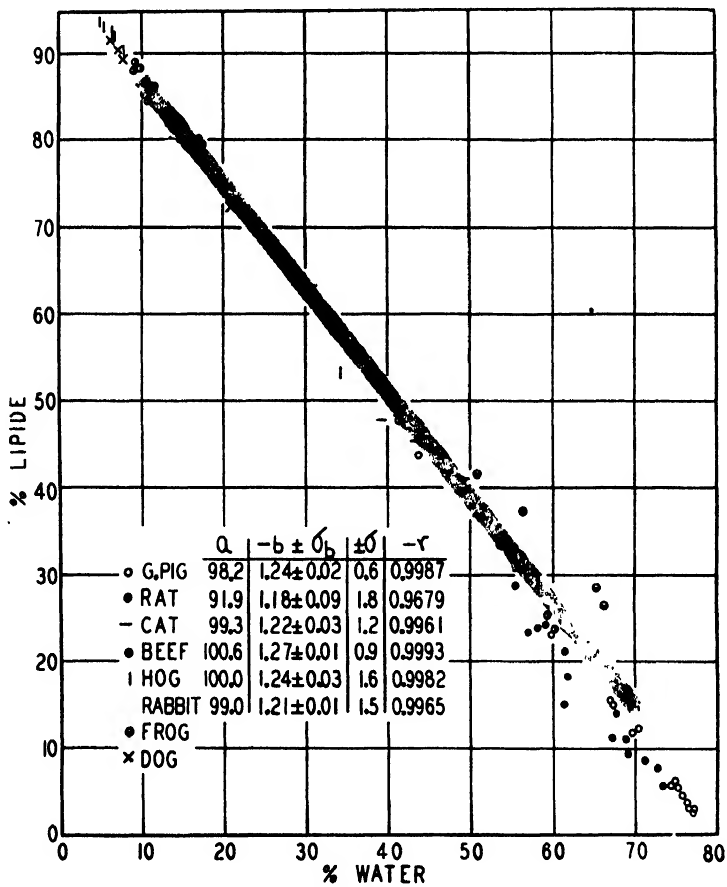

Fig. 2. Correlation of lipide and water in normal bone marrow. The values in the insert are for the equation, $L = a + (b \pm \sigma_b)W$ with a standard error of estimate, $\pm \sigma$, and the correlation coefficient, r. The shaded area represents the composition of normal rabbit marrow, $\pm \sigma$ (1).

Results

The plotting of the values obtained for the major bone marrow constituents showed a correlation in all animals. Some differences were noted from the values previously recorded for rabbit marrow (1). The chief difference was in the limits of the compositions. The marrow of the small animals, guinea pigs and rats, was distributed at the active end of the graphs, and the variations with age were less marked. Analyses representing samples of marrow from the femurs of the different animals

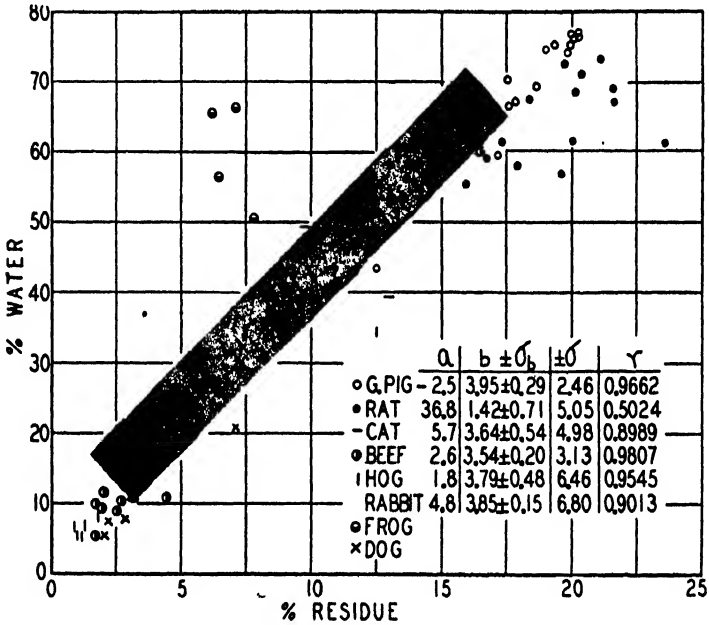

Fig. 3. Correlation of water and residue in normal bone marrow. The values in the insert are for the equation $W = a + (b \pm \sigma_b)R$ with a standard error of estimate, $\pm \sigma$, and the correlation coefficient, r. The shaded area represents the composition of normal rabbit marrow, $\pm \sigma$ (1).

are shown in Table I. With the larger animals, the marrow from the long bones showed a considerable decrease in activity with age, and all samples showed a distribution towards the inactive end of the scale. The differences are further shown in Figs. 1–4, where, for comparison, the line in Fig. 1 and the shaded areas in Figs. 2–4 represent the composition of normal rabbit marrow (1). The guinea pig marrow shows a tendency to deviate from the line given for the rabbit marrow, but this

deviation is hardly significant. The deviation of the rat marrow is more significant. In both cases, less water is associated with a unit weight of residue. In the values given in the legend of Fig. 1, *a* and *b* represent the g. H_2O associated with each gram of lipide and residue, respectively. The guinea pig, rat, and beef marrows have a significantly smaller

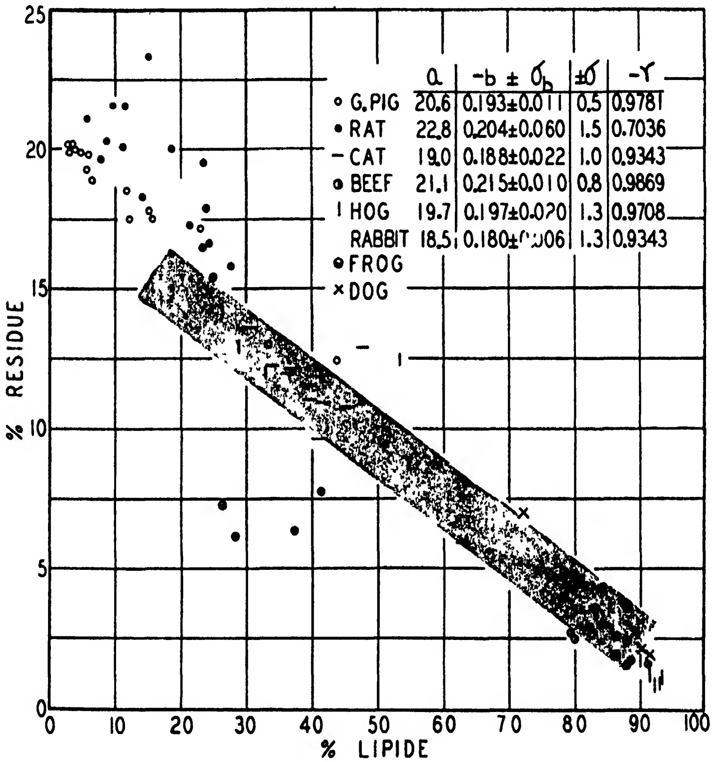

FIG. 4. Correlation of residue and lipide in normal bone marrow. The values in the insert are for the equation, $R = a + (b \pm \sigma_b)L$, with a standard error of estimate, $\pm \sigma$, and the correlation coefficient, r. The shaded area represents the composition of normal rabbit marrow, $\pm \sigma$ (1).

amount of water associated with the residue than in the case of the rabbit marrow. In no case was water associated with the lipide as the values for *a* in Fig. 1 are not significantly different from zero. The frog marrow differed from all the others. An insufficient number of samples were available to permit correlation analysis, but the points are so

located as to indicate a dilution with water. The points in Fig. 2 indicate that the lipide and water components of the frog marrow vary inversely with one another, but the other analyses tend to show a relatively constant residue and nitrogen content.

Correlation of Nitrogen Components

The residue showed a positive correlation with the nitrogen content of the marrow, and, in all cases, the regression lines fell within one standard deviation of the line previously given for normal rabbit marrow (1), and so are not included in this paper. The lines differed from one another in range of concentrations only.

The regressions of the lipide with the log of the lipide nitrogen showed a negative correlation in all cases (Fig. 5). Thus, there is relatively less nitrogen associated with the lipide in the inactive marrows. The hog and guinea pig marrows showed a slightly better correlation when the log of the lipide concentration was correlated with the log of the lipide nitrogen, but not sufficiently better to include another graph. As shown by the values in the legend of Fig. 5 and the placement of the points, the rat and guinea pig marrows show significant differences in both the slopes and limits of the regression line from rabbit marrow.

The results of the analyses for various sulfur and phosphorus components and for non-protein nitrogen could not be correlated readily with the activity of the marrow as expressed by its water, lipide, and residue content. Within a given animal there was usually an increase in inorganic sulfate and phosphate, total sulfur and phosphorus, and non-protein nitrogen with an increased activity of the marrow. Between the various rabbits there was, however, a larger variation. Representative analyses are given in Table II. The lipide concentration may be obtained by subtracting the percentages of water and residue from 100.

Using the values found in this table, and calculating the results on a lipide-free or lipide-free-solid basis, somewhat more constant values can be obtained for certain of the constituents, but marked differences are still found between the various samples. On the lipide-free basis, the total nitrogen, sulfur, and phosphorus for 9 samples of active marrow from the humerus, femur (and proximal tibia) are: 2.90 (2.45–3.22), 0.28 (0.21–0.41), and 0.37 (0.20–0.52)%, respectively. On the lipide-free-solid basis the values are: 14.5 (12.8–15.7), 1.40 (1.28–1.85), and 1.83 (1.18–2.48)%, respectively.

A calculation of distribution of sulfur shows that 73.6 (68.3–83.0)% is present in the proteins. In the non-protein fraction the average distribution of sulfur is 50.6% inorganic sulfate, 6.5% ethereal sulfate, and 42.9% non-sulfate sulfur. A calculation of the various nitrogen, sulfur, and phosphorus ratios on the equivalent weight basis gives: P/S = 2.05 (1.38–2.97), N/P = 12.4 (9.2–18.2) and N/S = 24.3 (16.5–30.0). The ratio of inorganic-phosphate phosphorus to inorganic-sulfate sulfur on the same basis was more variable, 1.66–6.17. Additional

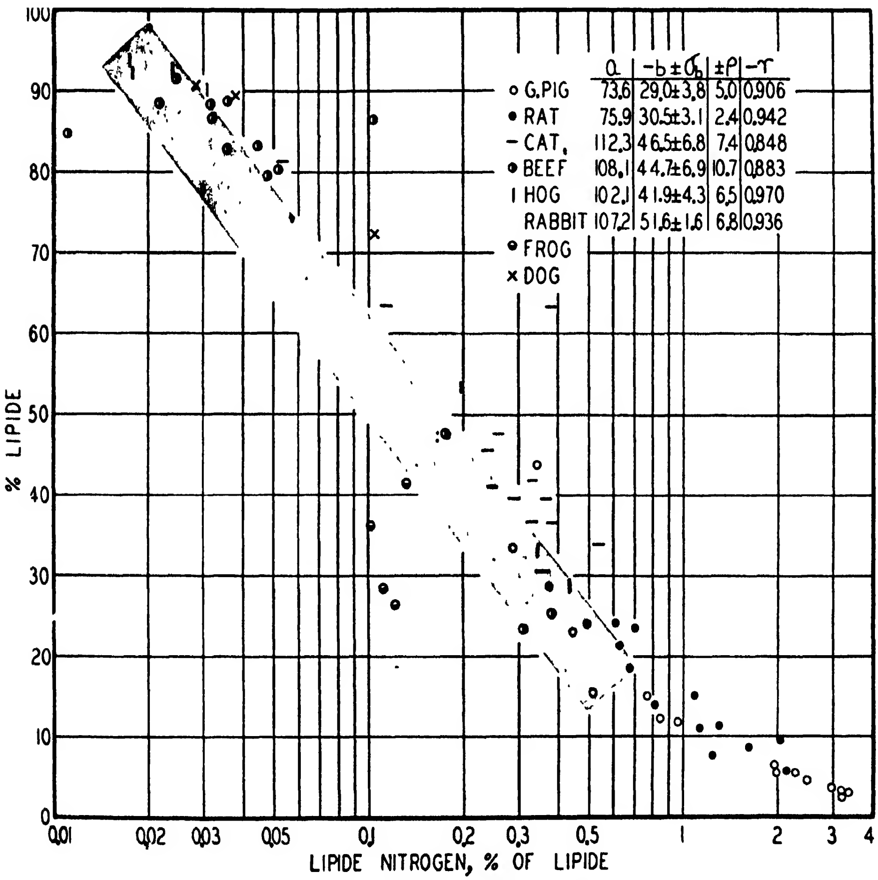

FIG. 5. Correlation of lipide with the log of its nitrogen content in normal bone marrow. The values in the insert are for the equation, $L = a + (b \pm \sigma_b) \log 100 N_L$ with a standard error of estimate, $\pm \sigma$, and the corelation coefficient, r. The shaded area represents the composition of normal rabbit marrow, $\pm \sigma$ (1).

TABLE II

Composition of Normal Rabbit Bone Marrow

Composition based on total marrow

Rabbit no	Water per cent	Residue per cent	Total sulfur mg.-% S	Non-protein sulfur			Phosphorus		Nitrogen		
				Inorg. SO_4 mg.-% S	Ethereal SO_4 mg.-% S	Organic S mg.-% S	Total mg.-% P	PO_4 mg.-% P	Total per cent N	Lipide mg.-% N	Non-protein mg.-% N
Humerus, femur, and proximal end of tibia											
1[a]	36.7	8.7	161	13.6	1.3	12.6	190	52.0	1.37	77	216
2	39.0	10.2	148	26.7	0.2	19.9	198	50.2	1.51	78	256
3[a]	41.3	12.3	220	18.7	10.6	18.1	206	58.5	1.58	88	368
4	43.0	11.4	134	21.6	1.8	19.2	202	39.0	1.53	61	216
5[a]	49.4	13.0	168	25.8	3.6	22.1	323	61.1	2.01	86	292
6	49.6	11.4	130	12.6	2.8	15.5	221	46.6	1.70	96	270
7[a]	50.9	11.7		38.6	4.3	7.3		51.8	1.79	131	310
8	52.4	13.0	166	22.5	3.0	14.5	267	47.9	1.92	96	295
9	59.7	14.3	194	29.2	1.8	21.6	171	31.0	2.11	85	284
10	63.5	13.3	176	29.8	0	23.0	157	57.6	1.88	87	314
Average[d]	48.3	11.9	166	22.3	2.8	18.5	218	49.3	1.73	84	279

TABLE II—*Continued*

Rabbit no.	Water *per cent*	Residue *per cent*	Total sulfur mg.-% S	Non-protein sulfur: Inorg. SO_4 mg.-% S	Non-protein sulfur: Ethereal SO_4 mg.-% S	Non-protein sulfur: Organic S mg.-% S	Phosphorus: Total mg.-% P	Phosphorus: PO_4 mg.-% P	Nitrogen: Total *per cent* N	Nitrogen: Lipide mg.-% N	Nitrogen: Non-protein mg.-% N
Ribs											
2	46.0	13.4	217	25.3			310	56.0	1.94	103	191
1	52.0	13.3	149	18.4			287	78.6	2.10	104	263
7	54.1	15.7		58.3				51.8	2.35	143	399
Radius, ulna, and distal end of tibia											
5	6.6	1.8	40	6.4			170	40.5	0.39	38	48
2	14.7	3.2	52	6.0	0	5.7	72		0.49	45	95
1	15.8	3.5	46	5.9			46	24.8	0.57	44	49
7	15.1	3.7		19.5				18.0	0.54	85	75
Beef[b]	63.8	13.7	131	5.6	1.9	6.5	169	64.0	1.92	77	187
Cat[c]	51.4	12.0		3.5	0	18.1		49.1	1.67	140	213

[a] Humerus and femur only.
[b] Marrow from a pocket of active marrow in the femur of an older bull.
[c] Marrow from humerus, femur and tibia.
[d] Rabbit no. 7 not included in average.

relationships can readily be calculated from the values given in Table II.

A sample of beef and cat marrow is included in Table II for comparison. The most marked difference is in the low values obtained for the inorganic sulfate content. Of several dog tissues analyzed, Denis and Leche (11) found the liver to have the largest concentration of total sulfate, 20–21 mg./100 g. The values given for rabbit bone marrow are of the same order of magnitude, and would be somewhat higher if both results were calculated on a lipide-free basis.

Discussion

The composition of many tissues can be expressed by a point, and variations from the average taken as deviations from normal. Where three or more variable major components are present, the normal variation is no longer expressible by an average, unless one of the variables is neglected. This is frequently done by expressing the composition on a fat-free basis. A more accurate picture, however, is obtained by calculating the correlation between the components and expressing the composition by a line. This method was applied by Fenn and Haege in a study of the composition of liver (12). It is of special importance in the study of the chemistry of bone marrow because of the great variation in the content of the three major components, water, lipide, and protein. Not only is the variation pronounced in different animals, but is very marked within a given animal, depending upon the bone from which it is removed. In all cases studied, the major components of the marrow showed a definite correlation to one another. Deviations from normal take the form of a shift in the limits of the line with the same coefficients, a change in the coefficients, or both. All types of changes have been found in pathologic marrow, and will be reported in other papers.

The inability to obtain a correlation between the lesser components and the activity of the marrow may, in part, be due to the unavailability of a sufficient number of samples from the same animal. Within a given animal the nitrogen, sulfur, and phosphorus usually increased with increased activity of the marrow. Marrows of similar water, lipide, and residue concentrations, taken from different animals, showed variable amounts of the lesser components. The relationship was such that high values of these components were associated with active marrow, but somewhat lower values did not necessarily indicate a less active marrow. This is similar to the relationship found by McCoy and

Schultze (4) for the hemoglobin concentration of animals recovering from anemia. The variability of the values may well indicate differences in the manufacture, storage, and discharge of the cellular elements.

Summary

There is a direct linear correlation between the water and residue (lipide-free solids) and an inverse linear correlation between these two components and the lipide content of marrow from guinea pigs, rats, cats, beef, hogs, and rabbits. The rat, guinea pig, and beef marrow had a relatively smaller amount of water associated with the residue than did that from rabbits. In no case was a significant amount of water associated with the lipide.

Samples of frog marrow have a relatively higher concentration of water than the marrow from the other animals studied.

High concentrations of total sulfur, phosphorus, non-protein nitrogen, and inorganic sulfate and phosphate are found in the active marrow samples of rabbits, but somewhat lower values for these constituents do not necessarily indicate a less active marrow. The variations found may be due to differences in the rate of manufacture and liberation of the cellular elements.

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Chemical Composition of Irradiated Bone Marrow

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Introduction

The literature on the cellular changes of bone marrow resulting from irradiation was recently reviewed by Warren and Dunlap (1). There is, however, little information on the chemical changes following irradiation of marrow. In this paper, the composition of marrow was determined at various intervals following the administration of a uniform dose of X-rays over the legs on the right side, and compared with unirradiated marrow removed from similar bones of the left legs.

Experimental

Rabbits over 4 months old were anesthetized with nembutal and the desired area irradiated with 3000 r of unfiltered X-ray delivered in a field of 380 $cm.^2$, at the rate of 160 r/min. The animals were killed at 1–32 day intervals following the irradiation. In most cases the right foreleg was irradiated with the rest of the body covered by 1.5 mm. of lead. In these animals the left foreleg was used as the control to measure the changes taking place in the irradiated limb, and the marrow of the hind legs was used to determine the effect of sampling. The marrow samples were analyzed for the major constituents as previously described (2). For the more complete analyses, the foreleg and the hind leg on the right side were irradiated separately and an aliquot of the combined humerus and femur from this side compared with a similar aliquot from the left side. Inactive marrow samples from the radius, ulna, and distal tibia were also compared, but, because of the relatively small changes in their composition, most of these results are not included. Aliquot samples were analyzed for the major components (2) and for the nitrogen, sulfur, and phosphorus distribution (3). The terms X-rayed, control, and normal marrow are used in connection with the composition of the irradiated and non-irradiated marrow from the same animal and the composition of normal rabbit marrow as previously reported (2,3), respectively. Representative samples of marrow were used for histologic examination.

Results

The changes in the gross composition of rabbit bone marrow following irradiation are summarized in Table I, and representative analyses

including the nitrogen, sulfur, and phosphorus components are given in Table II. At one day following irradiation, when the marrow showed a slight decrease in mature cells, no significant changes were noted in the gross composition, but the inorganic sulfate had decreased about 25%. With longer periods after radiation a marked increase in the lipide and decrease in the water, residue, and total and lipide nitrogen was found. Of the lesser components, the most marked changes were decreases in inorganic sulfate and non-protein nitrogen with slightly smaller decreases in the non-protein organic sulfur. The results on the inorganic phosphate determinations were somewhat irregular, but the analysis of a larger group of data than is given in Table II indicated

TABLE I

Changes from Control in the Percentage Composition of Active Bone Marrow Following 3000 r of X-Ray

Days after X-ray	No. of samples	Difference in per cent total marrow				Difference in per cent of lipide	Histologic changes
		Water	Lipide	Residue	Total nitrogen	Lipide nitrogen	
1	3	1.8	−2.7	0.9	0.21	0.023	Decrease in mature forms
2	5	−1.5	5.6	−4.1	−0.62	−0.067	Marked hypoplasia and nuclear degeneration
3	2	−4.4	9.3	−4.9	−0.97	−0.247	
4	5	−1.1	4.6	−3.6	−0.63	−0.068	
8	12	−6.2	9.6	−3.5	−0.58	−0.065	
S. D.[a]		±4.03	±5.34	±1.47	±0.229	±0.042	
t[b]		4.30	3.16	8.55	9.1	3.64	
P[b]		0.001	0.005	0.001	0.001	0.001	
16	4	−10.0	12.4	−2.5	−0.39	−0.066	Decrease in cellularity to no change
24	4	−6.2	8.8	−2.6	−0.48	−0.059	
32	7	−7.8	11.3	−3.5	−0.49	−0.062	
Non-irradiated hind legs	15	−0.57	0.62	−0.04	−0.004	−0.016	
S. D.[a]		±2.7	±3.23	±0.56	±0.088	±0.045	

[a] Standard deviation = $\sqrt{\dfrac{\Sigma x^2 - \bar{x}\Sigma x}{n-1}}$.

[b] t and P—see Ref. 4. Compared with hind legs.

TABLE II

Composition of Irradiated and Non-Irradiated Humerus and Femur Rabbit Marrow, 3000 r

Days after X-ray	Marrow	Water	Residue	Non-protein sulfur			Phosphate mg.-% P	Nitrogen		
				Inorg. SO_4 mg.-% S	Ether SO_4 mg.-% S	Organic S mg.-% S		Total N	Lipide mg.-% N	Non-protein mg.-% N
		per cent	*per cent*					*per cent*		
0	C[a]	35.5	10.5	16.3	2.6	14.4	51.0	1.52	80	126
	C	35.8	9.9	15.8	2.6	15.8	49.8	1.51	90	148
1	X[a]	43.1	10.4	12.3	0.5	11.8	32.6	1.47	73	113
	C	41.5		16.1	2.2	14.1	36.0			141
2	X	38.5	8.4	9.4	0.5	16.4	35.3	1.33	99	131
	C	39.5	11.3	19.0	2.8	26.0	66.4	1.54	108	251
4	X	40.7	9.0	2.9	0.5	4.8	103.5	1.18	90	92
	C	46.0	13.2	20.0	1.6		83.0	1.89	117	251
8	X	39.7	6.4	4.4	0.9	10.6	25.0		37	64
	C	40.1	8.1	9.9	1.2	17.6	45.8		66	94
16	X	23.6	6.7	9.0	1.4	16.1	56.5	1.02	100	128
	C	27.9	8.0	15.8	1.0	24.2	43.1	1.23	118	204
32	X	43.5		9.8	0.4	18.7	63.0			143
	C	51.4	13.3	19.1	2.3	20.1	56.2	1.96	87	206
4[b]	X	15.1	5.7	2.3			79.6	0.73	84	35
	C	16.7	7.9	6.1			50.5	1.08	162	99

[a] "C" and "X" represent Control and X-rayed marrows, respectively.
[b] Radius and ulna.

that the inorganic phosphate frequently showed a decline during the first 8 days and thereafter an increase over the control marrow. The smaller change in phosphate is also shown in the ratio of inorganic-phosphate phosphorus to inorganic-sulfate sulfur calculated from Table II on an equivalent weight basis. In many of the cases, this ratio for the irradiate marrow is more than double that of the control marrow, for example, in the samples 32 days after irradiation, the vlaues are 10.03 and 4.57, respectively. The distribution of the non-protein sulfur

components, showed a smaller percentage inorganic sulfate in the irradiated marrow in most cases.

The changes noted above were found up to 32 days following irradiation. They persisted even though, in some cases, little or no difference could be found in the histologic picture. The regeneration was not uniform in the different animals, which is consistent with the variation in response of individuals to X-ray, as found by others (1).

The increase in lipide and decrease in percentage composition of the other constituents is consistent with a decrease in cellularity of the marrow. The greatest number of values were obtained for the 8-day regenerated marrow. The significance P of the changes was calculated for these samples (4) (Table I). The likelihood of any *one* average difference occuring by chance is of the order of 1 in 1000.

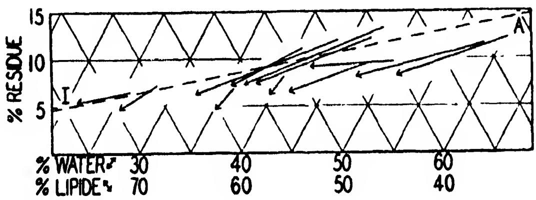

FIG. 1. Composition of irradiated and non-irradiated bone marrow from humerus 8 days after irradiation, plotted on triangular coordinates. The broken line represents normal marrow. The irradiated and non-irradiated marrow from similar sites in the same animal are shown by the arrowed and blank ends, respectively, of the shorter lines. The multiple correlation equation for the X-rayed marrow is:

$$W = (0.15 \pm 0.09)L + (6.81 \pm 0.72)R,$$

and for the control,

$$W = (0.026 \pm 0.12)L + (1.43 \pm 0.52)R.$$

Standard errors of estimate of the equations are 5.00 and 7.83, respectively.

Further analysis of the water, lipide, and residue composition of the irradiated marrow indicates changes other than a simple reduction in activity. In Fig. 1, where the composition of the 8-day regenerated marrow is plotted on triangular coordinates, and compared with the corresponding control marrow, some of the changes tend to parallel the line for the normal marrow. This change is consistent with a simple decrease in activity. The majority of the lines, however, deviate from this, in that there is relatively less residue in the irradiated marrow. This is further shown by the coefficients of the residue in the multiple correlation equations, Fig. 1. The coefficient of R, g. H_2O/g. residue,

for the non-irradiated control marrow is 4.43 ± 0.52, and for the irradiated 6.81 ± 0.72. In neither case was a significant amount of water associated with the lipide.

In Figs. 2–4, the simple correlations of the major components are graphed. The most pronounced change is in the shift of the activity of

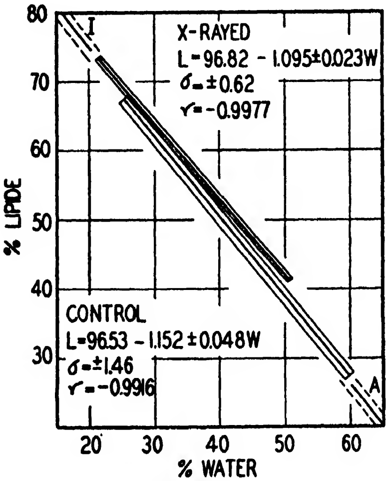

FIG. 2. Correlation of lipide and water in irradiated and non-irradiated bone marrow 8 days after irradiation. The line A-I, if connected, is the regression line of normal rabbit marrow; the short dashes being one standard error of estimate from the solid lines. A and I representing the active and inactive ends of the marrow scale, respectively. The standard errors of estimate are shown by the sides of the rectangles, and the ranges in composition by the lengths.

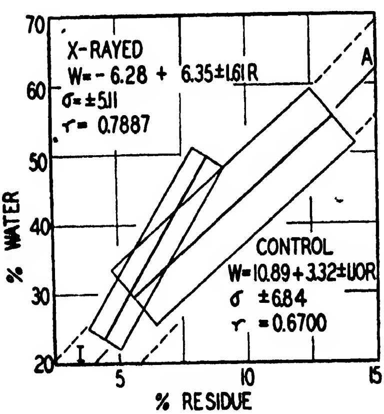

FIG. 3. Correlation of water and residue in irradiated and non-irradiated bone marrow 8 days after irradiation. *Cf.* Fig. 2.

the irradiated marrow toward the inactive end of the curves. The loss of residue is relatively greater than the loss of water, Fig. 3. The slopes of the curves and the position of the limits show the deviations from normal to be greatest for the marrow samples normally most active. Marked decreases in inorganic sulfate and non-protein nitrogen were

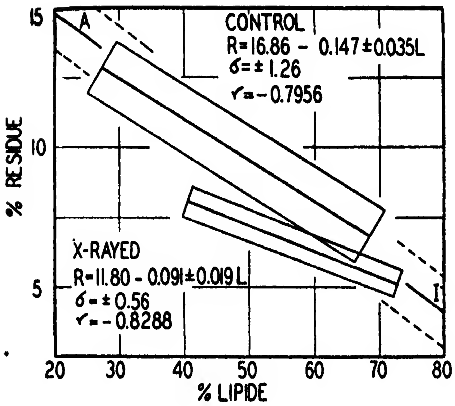

FIG. 4. Correlation of residue and lipide in irradiated and non-irradiated bone marrow 8 days after irradiation. *Cf.* Fig. 2.

found in the inactive marrow, of the radius and ulna (Table II), even though the changes in the major components were small.

The equations for the regressions of total nitrogen on residue and for the log of lipide nitrogen on lipide did not deviate significantly from normal as the calculated lines for the irradiated marrow fell within one standard error of estimate of the normal (2). There is, however, a shift of the irradiated samples toward the inactive end of the curves. The magnitude of this shift can be seen in Table I.

The analysis of the protein-free filtrates of the serum of these animals showed no abnormal changes in the nitrogen, phosphorus, or sulfur content. In view of previous work (1), measurable changes in the blood would not be expected to result from the irradiation of a small area.

DISCUSSION

Many of the chemical changes due to irradiation have been deduced from observations on blood or on urine (1). The analyses recorded in this paper show the magnitude of the effect of irradiation on the bone marrow itself. The increased amount of water associated with a unit weight of residue in the irradiated marrow is consistent with observed edema in and around the cells of the irradiated area (1). An increased urinary excretion of non-protein nitrogen is usually explained as a result of the destruction of protein (1), but the marked decrease in the non-protein nitrogen of the irradiated marrow would indicate that at least a portion of it is derived from the non-protein nitrogen of the tissue. The relatively great changes in the inorganic sulfate and non-protein organic sulfur would indicate that the various sulfur compounds play an important role in the hemopoietic function of bone marrow. The decrease in inorganic phosphate, followed by an increase after about 8

days, is in line with the observation that, "Alterations in the total phosphorus level is by no means a constant finding after irradiation, and when present is usually of brief duration." (1).

Summary

Marrow, following 3000 r of X-ray, was analyzed at intervals up to 32 days after irradiation and compared with non-irradiated marrow of the same animals. The irradiated marrow showed an increase in lipide content and decrease in water, residue (lipide-free solids), non-protein sulfur fractions, and total, lipide, and non-protein nitrogen. The changes in composition were greatest in marrow normally most active. A relatively greater amount of water was associated with the residue of the irradiated marrow.

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Ultracentrifugal Studies on Some Porcine Plasma Protein Fractions

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Introduction

In view of the availability of some protein fractions from porcine plasma, a study of the ultracentrifugal properties of some of the protein fractions was undertaken.

The freshly collected citrated porcine plasma was fractionated in the Armour Laboratories according to procedures patterned after those developed by Cohn *et al.* (1,2). Only those fractions were studied which had been examined electrophoretically. The following fractions were studied: Fraction II P-2 (a fairly electrophoretically homogeneous preparation of γ-globulin consisting of one resolvable component), Fraction III-1 P-2 (a mixture which consisted of 57% of a protein with the mobility of γ-globulin and 43% of a protein with the mobility of β-globulin), and Fraction V P-5 (a mixture which consisted of 84% of a protein with the mobility of albumin, 8% of a protein with the mobility of α-globulin, 6% of a protein with the mobility of β-globulin, and 2% of a protein with the mobility of γ-globulin). The electrophoretic analyses were furnished by the Armour Laboratories.

Experimental

All of the experiments were carried out on the Svedberg oil turbine velocity centrifuge. A speed of 59,000 r.p.m. was used. The Lamm Scale method for observing the sedimenting boundaries was used throughout the investigation. All the preparations were dissolved in 0.2 *M* NaCl. The preparations were stored in the lyophilized form under refrigeration. Sedimentation runs were made at 10 different protein concentrations, ranging from 0.15% to 5.00% based on the dry weight of the preparation; thus the variation of the sedimentation constant with protein concentration could be observed. The treatment of the ultracentrifugal data was the same as Pedersen's (3).

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Results

Fraction II (*γ-globulin*). Fig. 1 shows the sedimentation diagram for Fraction II in a 0.5% solution after running for 80 min. The ordinate, Z, represents the scale line displacement and is proportional to the concentration gradient, while the abscissa, x, is the scale reading for the test run and is proportional to the distance from the axis of rotation, meniscus, *etc.* There is evidence for a small amount of heavier material as well as a very small amount of lighter material. The preparation is, however, predominately one component. The curve is symmetrical.

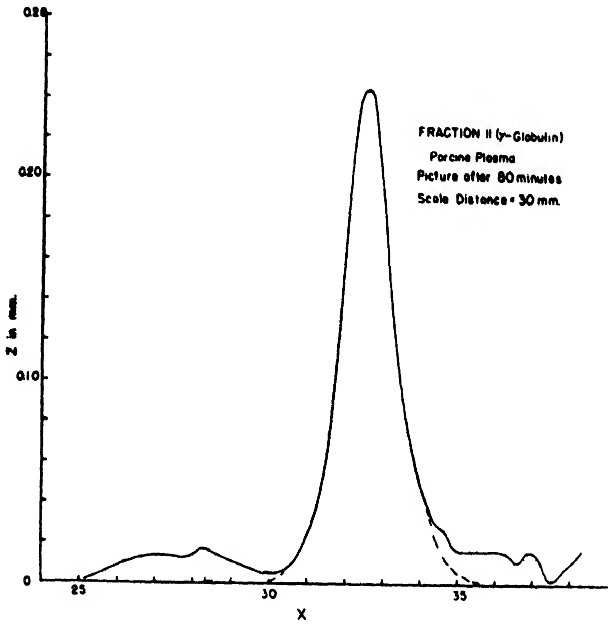

FIG. 1. Sedimentation diagram for Fraction II.

The equation for the line of regression of S_{20} on concentration, c, as calculated by the method of least squares and the correlation coefficient, are given in Table I. Concentrations in terms of Δn, the refractive index increment, are calculated from the diagram by the method of Pedersen (4). The equation for the line of regression of S_{20} on Δn, obtained by the method of least squares, and the correlation coefficient are given in Table I.

Fraction III-1. The sedimentation diagram for a 0.5% solution after sedimenting for 50 min. is shown in Fig. 2. The preparation consists

TABLE 1

Protein fraction	Line of regression for S_{20} on concentration	Correlation coefficient r	Line of regression for S_{20} on Δn	Correlation coefficient r
II	$S_{20}=7.28-0.39c$	-0.949	$S_{20}=7.25-0.0025\ \Delta n$	-0.951
III-1 1st comp.	$S_{20}=40.46-37c$	-0.895	$S_{20}=44.72-0.83\ \Delta n$	-0.628
2nd comp.	$S_{20}=22.64-1.68c$	-0.932	$S_{20}=23.19-0.22\ \Delta n$	-0.925
3rd comp.	$S_{20}=7.28-0.18c$	-0.973	$S_{20}=7.28-0.0020\ \Delta n$	-0.967
V Albumin	$S_{20}=4.68-0.18c$	-0.983	$S_{20}=4.67-0.0016\ \Delta n$	-0.982
Heavy component	$S_{20}=7.33-0.46c$	-0.992	$S_{20}=7.50-0.019\ \Delta n$	-0.967

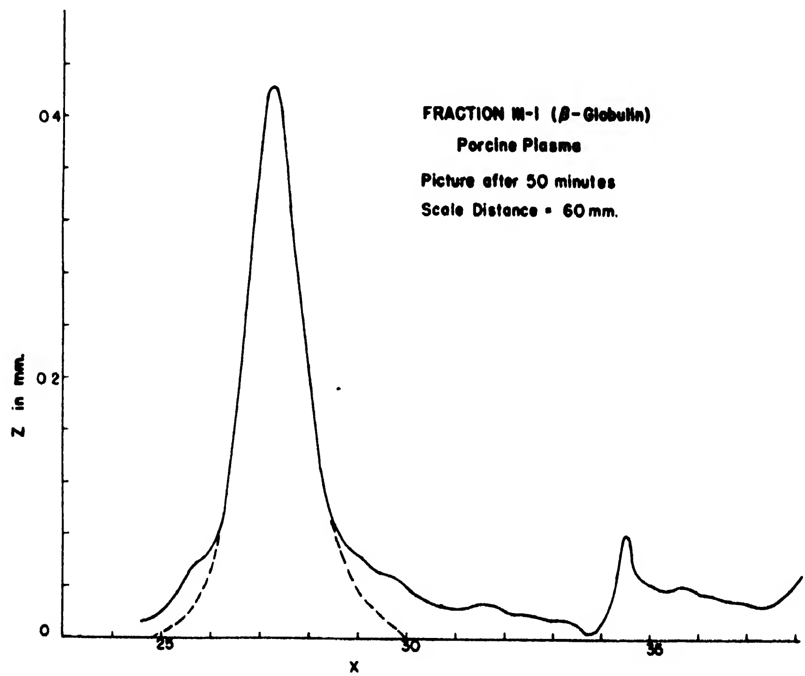

FIG. 2. Sedimentation diagram for Fraction III-1.

essentially of one major component with at least 2 faster sedimenting components and a slower sedimenting component in small concentrations. The equations of the regression lines of S_{20} on c and on Δn are given in Table I, together with the respective correlation coefficients. The concentration values in the equations of Table I represent total

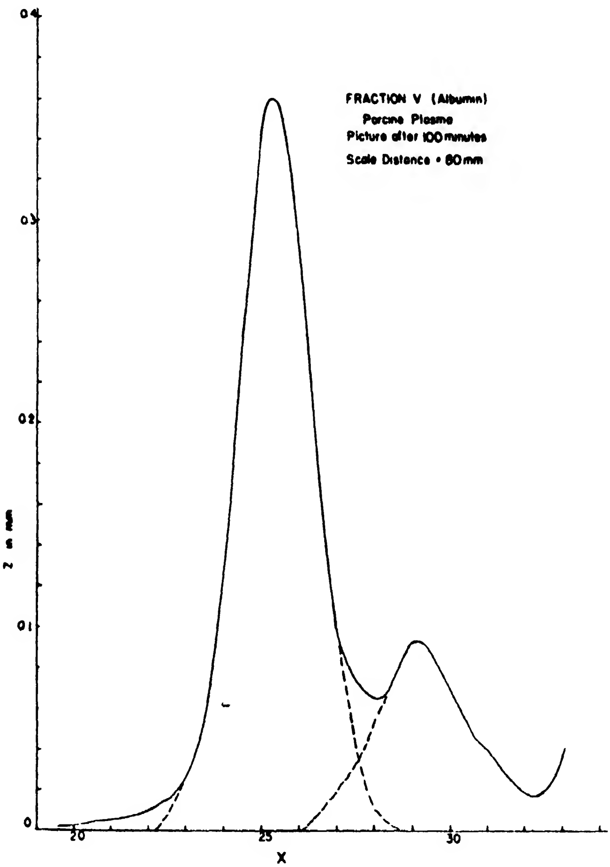

FIG. 3. Sedimentation diagram for Fraction V.

concentration based on the preparation as weighed out in dry form, and do not represent the exact concentration of each component. The absolute concentration would of necessity be the total concentration of the protein multiplied by the percentage composition of each component in the mixture.

Fraction V. The sedimentation diagram for a 0.5% solution of Fraction V after running 100 min. is given in Fig. 3. The preparation predominates in albumin, but has with it about 20% of a heavier sedimenting protein. The equations for the regression lines of S_{20} on c and on Δn are given in Table I, together with the respective correlation coefficients. The concentration values in the equations of Table I represent total concentration of the preparation as weighed out in dry form, and do not represent the concentration of each component. As in the case of Fraction III-1, the absolute concentration would of

TABLE II

Summary of Data

Concentration in per cent	Fraction II		Fraction III-1						Fraction V			
	S_{20}	Δn	1st component		2nd component		3rd component		Albumin		Heavy Component	
			S_{20}	Δn	S_{20}	Δn	S_{20}	Δn	S_{20}	Δn	S_{20}	Δn
0.15	7.48	29	41.26		22.21	3	7.25	14				
0.25	6.96	37			24.87		7.31	26	4.70	26	7.37	9
0.50	6.92	80	40.02		21.63	8	7.11	17	4.53	54		
0.75	6.92	113	38.65	9	20.56	9	7.13	72	4.55	92	6.92	25
1.00	6.89	147	35.20		19.77	19	7.17	87	4.51	108	6.88	34
1.50	6.86	219	30.94	16	20.16	17	7.07	129	4.42	156	6.67	44
2.00	6.57	285	30.81	12	18.24	24	6.84	188	4.25	215	6.29	71
3.00	6.32	420	36.30	14	17.84	29	6.82	229	4.23	300	5.93	98
4.00	5.33	610	22.53		15.71		6.49	324	3.89	429	5.43	115
5.00	5.46	812	22.73		14.90	30	6.46	461	3.79	578	5.17	103

necessity be the total concentration of the preparation multiplied by the percentage composition of each of the two components in the preparation.

Table II gives a summary of the Δn data.

Discussion

There is good agreement between the S_{20} values for Fraction II extrapolated to zero concentration and zero Δn; namely 7.28 S and 7.25 S, respectively. The slopes of the two regression lines have the expected difference, inasmuch as a constant relating index of refraction

to concentration would be the ratio of the two slopes. The correlation coefficients 0.949 and 0.951 indicate well the straight-line relationship between S_{20} and concentration or Δn.

Fraction III-1 presents a mixture of proteins with a definite preponderance of one component. The diagram of Fig. 2 shows the main component and one heavier component. The heaviest component has already sedimented to the bottom of the cell and is not visible in the diagram. This component exhibits rather high concentration dependence, as judged by the slope of the regression line. This dependence is probably due in part to the viscosity of the solution because of the presence of the other proteins, and in part may indicate a long filamentous molecule. This heavy component may indicate a denatured portion of the fraction. The S_{20} values extrapolated to zero concentration and zero Δn agree rather well in view of the fact that there was a limited amount of Δn data. The limited amount of Δn data is reflected in the lower correlation coefficient, 0.628. The r value for concentration, 0.895, is more substantial.

The second component, probably the 20-component of Pedersen (3), exhibits rather high concentration dependence as judged by the slope of the regression line. Here, a long filamentous molecule is suggested, as well as the other factors discussed under the previous component. There is good agreement between the S_{20} values extrapolated to zero concentration and zero Δn. The correlation coefficients, 0.932 and 0.925, indicate the straight-line relationship.

The third component or the main component includes the greatest part of the protein. There is rather low concentration dependence. The S_{20} value extrapolated to zero concentration agrees well with the value extrapolated to zero Δn. The S_{20} values are the same as those for Fraction II. From the electrophoretic analysis, one would conclude that this component is made up of both β- and γ-globulins, both of which have about the same sedimentation constant. The r values are good and support the straight-line relationship.

The diagram in Fig. 2 indicates the presence of a lighter component and is better resolved as the sedimentation progresses, but not well enough resolved to be quantitatively considered.

Fraction V represents albumin contaminated with a heavier component. It has so far not been possible to obtain a pure albumin. The preparation described here represents the best that has been obtained in any quantity. The S_{20} values extrapolated to zero concentration and

zero Δn agree well for the albumin, the lighter, and the main component. The correlation coefficients, 0.983 and 0.982, show the straight-line relationship.

The heavy component shows fairly good agreement between the S_{20} values extrapolated to zero concentration and zero Δn. The values are close to those for the β- and γ- globulins. The r values, 0.992 and 0.967, respectively, support the straight-line relationship.

Acknowledgments

The author is indebted to Professor The Svedberg for his kindness in making available the facilities of the Institute for this work, and to The Armour Laboratories, Chicago, for making the plasma proteins available, as well as for other help. The author is grateful to Laborator Kai O. Pedersen for his advice and help. Many thanks are extended to Mr. Evald Hellman, who, together with his staff, carried out most of the calculations on the experimental data. To the technicians who operate the ultracentrifuge, thanks and appreciation are extended for their cooperation.

Summary

An ultracentrifugal study on a series of protein fractions from porcine plasma has been made. By extrapolating to zero concentration the straightline formed by plotting sedimentation against concentration, the sedimentation constants at infinite dilution have been determined. The following values were obtained: Fraction II, γ-globulin, 7.25 7.28 S; Fraction III-1, a mixture of β- and γ-globulins, 40.46–44.72 S, 22.64–23.19 S, and 7.28 S; Fraction V, an impure albumin, 4.67–4.68 S and 7.33–7.50 S. The properties and composition of these fractions were discussed.

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Occurrence of an Unidentified Rat Growth Factor in Cottonseed Meal

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Introduction

In a recent paper, Register *et al.* (1) described an improved rat assay procedure for a growth factor present in liver extract. The corn-soybean ration used in these tests, even though well fortified with vitamins and minerals, was found to be inadequate for normal rat growth. Including either desiccated thyroid or iodinated casein at an appropriate level depressed the growth rates of the animals still further. A factor(s) present in commercial pernicious anemia liver extract was effective in restoring the rat growth to a more normal rate. Subsequent studies by Register *et al.* (2) have demonstrated that crystalline vitamin B_{12} will give a quantitative response in this assay.

Evidence presented in this report indicates that cottonseed meal contains the rat growth factor as contrasted to soybean meal which does not. This observation is in accordance with that of Zucker and Zucker (3) who have reported the existence of a lactation factor in cottonseed meal.

Experimental

Four test diets were prepared and fed to 4 groups of male weanling rats, 8 rats per group. Gp. 1 received the basic corn-soybean ration,[1] Gp. 2 received the corn-soybean diet plus 0.25% desiccated thyroid,[2] Gp. 3 received the same diet as Gp. 1, except for the substitution of hydraulic cottonseed meal for soybean meal, and Gp. 4 received the same diet as Gp. 3 plus 0.25% desiccated thyroid. At the end of the fourth week (Fig. 1) the desiccated thyroid content of the diets fed Gp. 2 and 4 was increased to 0.4%. Starting the sixth week, Gp. 2 was subdivided into two groups of 4 rats each.

[1] This ration is the same as that of Register *et al.* (1) except for the inclusion of 1% A and D oil (3000 I. U. of A, 400 I. U. of D/g.) at the expense of the corn and soybean meal.

[2] Parke-Davis desiccated thyroid containing 0.3% iodine. This preparation is 50% stronger than U. S. P.

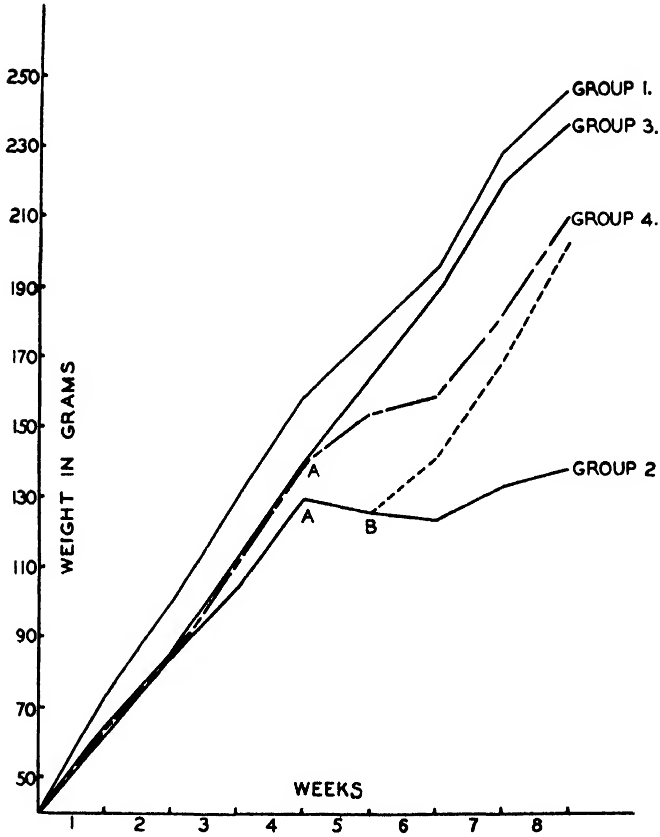

FIG. 1. Growth rates of rats receiving soybean and cottonseed meal diets with and without added desiccated thyroid.

Gp. 1, Corn-soybean basal diet; Gp. 2, Corn-soybean basal diet plus 0.25% desiccated thyroid; Gp. 3, Corn-cottonseed basal diet; Gp. 4, Corn-cottonseed basal diet plus 0.25% desiccated thyroid.

A. Desiccated thyroid content increased to 0.4% in diets fed gps. 2 and 4.

B. Gp. 2 subdivided and half (broken line) given daily injections of liver extract.

One subgroup remained on the desiccated thyroid diet as a negative control, whereas the other subgroup received additional supplements of 1 U. S. P. unit of liver extrect[3] per rat injected daily.

From Fig. 1, it can be seen that for the first 4 weeks of the experiment, 0.25% desiccated thyroid produced a marked decrease in the growth rates of rats receiving the corn-soybean diet (Gps. 1 and 2). Those rats receiving the cottonseed meal diet with 0.25% desiccated thyroid added (Gp. 4) grew as well as their controls on the

[3] Armour 15 U. S. P. units/cc.

unsupplemented cottonseed meal ration (Gp. 3). Elevating the level of desiccated thyroid to 0.4% produced a marked drop in the growth rates of rats receiving the corn-soybean diet and, to a much lesser extent, those animals receiving the corn-cottonseed meal diet. However, those rats receiving the cottonseed meal diet appeared to recover in part from the increased amount of desiccated thyroid and began to grow at a rate comparable to that of their controls (Gp. 3). The growth rates of rats receiving the corn-soybean diet plus 0.4% desiccated thyroid remained depressed. The subgroup which received liver extract as a supplement to the desiccated thyroid-corn-soybean diet demonstrated a marked weight gain, indicating that an actual deficiency had existed which could be overcome by supplying the missing factor.

Conclusions

Therefore, it may be concluded that hydraulic cottonseed meal contains activity for the rat growth factor. It is not certain whether this activity is due to the presence of vitamin B_{12} or to the existence of some other antithyroid substance in cottonseed meal. Nevertheless, considerable heat is produced in the preparation of hydraulic meal, and it would, therefore, appear that the factor is relatively heat stable. Experiments are now in progress to determine the effect of processing upon the content of this factor in cottonseed meal, and to ascertain the amount present in several composite samples.

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Stereochemical Configuration and Provitamin A Activity. VII. Neocryptoxanthin U

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Introduction

The *cis-trans* isomerization of cryptoxanthin, $C_{40}H_{55}OH$, has been studied by several authors (2,6,7,8,10), and recently the formation and differentiation of the neocryptoxanthins U, A, and B was observed on the Tswett column (9). The naturally-occurring all-*trans* isomer was found to show 56% of the biopotency of that of β-carotene (4), while the corresponding figure for neocryptoxanthin A (5) amounts to only 42%. Since we have now been able to obtain neocryptoxanthin U in analytically pure, crystalline state (2), some comparative assays of its provitamin A effect in the rat are reported below.

Experimental

The tests were made in the same manner as earlier (2,3,4), except that only male rats were used. The supplements were prepared at the start of the test; each test substance was stored in a number of separate bottles under CO_2 in the deep freeze. A new sample was used twice weekly. The desired dose was present in 0.1 ml. of Wesson oil which contained an added amount of 0.5% α-tocopherol (0.5 mg. per dose). No appreciable destruction of β-carotene, cryptoxanthin, or neocryptoxanthin U occurred after storing for one week in an ordinary refrigerator. In the deep freeze, the extinction values were identical with those of the original samples even after six weeks.

Results

Table I gives the data on the 107 rats used while the data from which the potency is calculated are given in Fig. 1.

[1] Née Mookerjee; Fellow of the Indian Government (Deputationist).

[2] Contribution No. 1288.

[3] Paper number 212 of the Department of Biochemistry and Nutrition, University of Southern California.

TABLE I

Summary Table Giving Body Weights and Total Gain in Weight for Male Rats Receiving β-Carotene, All-trans Cryptoxanthin, or Neocryptoxanthin U in Wesson Oil Containing 0.5% α-Tocopherol and for Negative Controls Receiving only Wesson Oil Containing 0.5% α-Tocopherol

Supplement	Dose per day	Number of rats	Average starting weight[a]	Average increase in body weight to following days					Average total gain	Average final weight	Number died	Calculated potency β-carotene =100
				5th	10th	15th	20th	25th				
	γ		g.	g.	g.	g.	g.	g.	g.	g.		
β-Carotene	0.5	12	103.3	−4.1	7.2 (11)	13.2	19.4	22.6	28.0 (10)	129.8	2	
	1.0	12	99.6	5.7	17.7	27.6	38.5	46.5	49.4	149.0	0	
All-*trans*-crypto-	0.75	12	101.4	2.9	10.5	15.4	18.9	24.5	26.5	127.9	0	63.4
xanthin	1.25	12	101.9	2.6	14.1	22.9	22.9	35.9	39.0	140.9	0	57.4
Neocryptoxanthin U	2.5	12	103.0	−2.7	4.8	9.3 (11)	15.4 (10)	20.1 (7)	19.6	124.0	5	25.8
	3.5	11	100.7	4.8	14.2 (10)	17.5	21.4 (9)	35.9 (8)	35.3	138.0	3	25.4
	4.5	12	101.8	0.2	14.1	23.5	32.7	49.8	51.0	154.8	1	30.1
Negative controls	0.0	12	100.6	−3.3	−6.8 (11)	−14.2 (8)	−19.6 (4)	−19.9 (3)	−11.0 (2)	82.0	10	

[a] Body weight at end of the depletion period and at the start of the assay period.

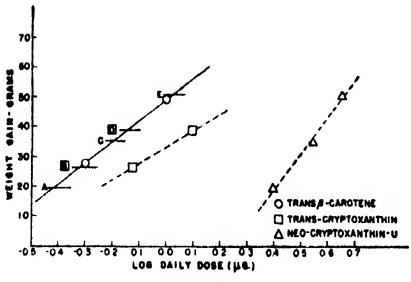

FIG. 1. Relationship of gain in weight to log of daily dosage of β-carotene, all-*trans*-cryptoxanthin and neocryptoxanthin U. Points A, C, and E represent the projection of the growth of rats receiving 2.5, 3.5, and 4.5 γ, respectively, daily of neocryptoxanthin U on the β-carotene curve while points B and D are the projections of the growth of rats receiving 0.75 and 1.25 γ, respectively, daily of all-trans-cryptoxanthin on the β-carotene curve.

The average provitamin A potency of all-*trans*-cryptoxanthin is 60% of that of β-carotene, which is in agreement with the value reported earlier (4) of 56%. The biopotency of neocryptoxanthin U averages 27% of that of β-carotene. This figure corresponds with a value of 45% of that of the all-*trans* form of this carotenol. The decrease of the activity lies roughly in the same range as that of neo-β-carotene U which has a potency of 38% of that of the all-*trans*-β-carotene (3).

SUMMARY

Neocryptoxanthin U, probably a mono-*cis* isomer, shows a provitamin A activity in the rat of 27% of that of all-*trans*-β-carotene, or 45% of that of all-*trans*-cryptoxanthin.

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Stereochemical Configuration and Provitamin A Activity. VIII. Pro-γ-Carotene (a Poly-*cis* Compound) and Its All-*trans* Isomer in the Rat

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Introduction

As is well known, the introduction of one or two *cis* double bonds into a naturally-occurring, all-*trans* carotenoid brings about a sharp reduction in the provitamin A potency (1,3,5,6,7). The only instance where an increase in provitamin A activity has been noted is in the case of a naturally-occurring poly-*cis* compound termed pro-γ-carotene as compared with all-*trans*-γ-carotene samples obtained from *Mimulus longiflorus*, Grant, or *Pyracantha angustifolia*, Schneid (4,2).

However, since the respective all-*trans*-γ-carotene preparations as obtained from different sources show definite divergencies; *e.g.*, in their melting points (8), it cannot be excluded that these are caused by minor variations which are of structural rather than stereochemical nature.

In order to decide in an unambiguous manner whether or not the provitamin A potency of a poly-*cis* compound may be equivalent to that of the corresponding all-*trans* form, we have now prepared an analytically pure, chromatographically homogeneous, crystalline sample of all-*trans*-γ-carotene by iodine catalysis of a fresh solution of pure crystals of the naturally-occurring pro-γ-carotene (*ex Pyracantha angustifolia*) (4,2). The comparative bioassays of these two provitamins which must be structurally identical and are only sterically different,

[1] Contribution No. 1289.

[2] Paper No. 214 of the Department of Biochemistry and Nutrition, University of Southern California.

are described below. Some experiments with a sample of all-*trans*-γ-carotene obtained from commercial tomato paste were also included.

The tests were carried out in the same manner as earlier (1–7), except that only male rats were used. The samples of supplement were prepared in Wesson oil containing 0.5% α-tocopherol at the start of the experiment and stored under CO_2 in a number of bottles in deep freeze. A new sample was used twice weekly. There was no appreciable destruction of the carotenoids during the tests, as determined spectroscopically.

Experimental

The all-*trans*-γ-carotene was prepared from pro-γ-carotene (*ex Pyracantha*) as follows.

One hundred fifty mg. of crystals in 80 ml. of petroleum ether (b.p. 60–70°C.) in a 100 ml. Pyrex volumetric flask were combined with 4.5 mg. of iodine in the same solvent. The flask was irradiated with a 400 watt, water-cooled, incandescent bulb from a distance of 10 cm. for 5 min. Then the solution was developed with petroleum ether containing 6% acetone on a calcium hydroxide + Celite (3:1) column (30 × 8 cm.) and the main upper zone of all-*trans*-γ-carotene was cut out and eluted with acetone + ethanol. (The paler neo zones were catalyzed again as described.) The combined solutions of all-*trans*-γ-carotene were rechromatographed on alumina + Celite (4:1; benzene + petroleum ether 3:2). The main pigment zone was cut out, eluted, transferred with water into petroleum ether, washed and dried. The filtered solution was then evaporated *in vacuo* to dryness and the residue was dissolved in the minimum amount of benzene. The all-*trans*-γ-carotene was crystallized out by adding dropwise absolute methanol (cooling). After two recrystallizations, the yield was 40 mg., m.p. 135–7°C. (corr.). $E^{mol.}_{1\,cm.} = 15.7 \times 10$ at 461 mμ, in hexane; and 14.2×10^4 at 473 mμ in Wesson oil.

Anal.: Calcd. for $C_{40}H_{56}$: C, 89.48; H, 10.52; found: C, 89.79; H, 10.38.

In a mixed chromatogram test there was no separation of all-*trans*-γ-carotene *ex* pro-γ-carotene and an authentic sample isolated from tomato paste or commercial carotene preparations.

Results

Two series of tests were carried out, but the results of only the second series are recorded in Table I.

The average potency of pro-γ-carotene was found to be 41% of that of all-*trans*-β-carotene. This result agrees well with the 44% value recorded earlier (4).

The provitamin A activity of the all-*trans*-γ-carotene prepared by iodine catalysis from the pro-γ-carotene was identical (42%) with that of this poly-*cis* isomer. A similar high result was also obtained with the all-*trans*-γ-carotene prepared from tomato paste where the two values give an excellent agreement at a figure of 47%. On the other hand, it is

apparent that the response of the sample of all-*trans*-γ-carotene used here is markedly superior to that of the product obtained from *Mimulus* or from *Pyracantha* where potencies of only 28 and 26% of the β-carotene values were found earlier.

TABLE I

Summary Table Giving Body Weights and Total Gain in Weight for Male Rats Receiving β-Carotene, All-trans-γ-Carotene (ex Pro-γ-Carotene and Tomato Paste), and Pro-γ-Carotene in Wesson Oil Containing 0.5% α-Tocopherol, and for Negative Controls Receiving only Wesson Oil Containing 0.5% α-Tocopherol

Supplement	Dose per day	Number of rats	Average starting weight[a]	Average total gain	Average final weight	Number died	Calculated potency (β-carotene = 100)
	γ		g.	g.	g.		
β-Carotene	0.5	11	103.3	17.5	121.1 (10)	1	
	1.0	13	106.7	46.5 (11)	153.5 (11)	2	
All-*trans*-γ-carotene (*ex* pro-γ-carotene)	1.2	13	105.8	18.5	124.4 (12)	1	43
	1.8	13	105.2	33.3	137.7 (12)	1	41
	2.4	13	105.7	47.5	153.2	0	42
Pro-γ-carotene	1.2	12	105.8	18.5	124.3	0	43
	1.8	14	105.1	30.7	135.8	0	38
	2.4	13	105.7	46.5	152.2	0	42
All-*trans*-γ-carotene (*ex* tomato paste)	1.2	13	107.5	22.5	129.9	0	47
	1.8	13	107.2	39.2	147.9 (12)	1	47
Negative controls	0.0	12	100.6	−11.0 (2)	82.0 (2)	10	—

[a] Body weight at the end of the depletion period and at the start of the assay period.

We cannot offer any clear explanation for these divergencies at the present time. However, considering the consistency of the new results which were obtained on the same strains of animals, we believe we have proved that there exist such *cis-trans* isomeric differences which do not

affect the biopotencies in the rat. The potencies in the several groups (starting from lowest dosage) were as follows: all-*trans*-γ-carotene (*ex* pro-γ) 42.7, 40.6, and 41.8; pro-γ-carotene, 42.7, 38.0, and 41.7; and all-*trans*-γ-carotene (*ex* tomato), 47.0 and 46.6. Moreover, 83% of of the group of negative controls lost weight or had died before the eleventh day and only two survived and these lost an average of 11.0 g. This would indicate that the dietary regime was vitamin A-free and that the rats used were sufficiently depleted of vitamin A at the start of the test. At least in this animal, the naturally-occurring poly-*cis* provitamin A, pro-γ-carotene is as potent as its poly-*trans* form, termed γ-carotene.

Summary

The earlier reports which showed that pro-γ-carotene has a biological potency in rats equal to 44% of that of β-carotene have been confirmed, since the new results are calculated at 41%. All-*trans*-γ-carotene obtained by iodine catalysis of naturally-occurring pro-γ-carotene was found to have a provitamin A activity of 42%. It has been demonstrated that, in this case, a poly-*cis* provitamin A and its poly-*trans* stereoisomer are biologically equivalent in the rat.

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Comparative Study of the Glycolysis and ATP-ase Activity in Tissue Homogenates [1]

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In some foregoing papers (1,2) the balanced activities of the enzymes of the glycolytic cycle were studied. It was, for instance, shown that the high rate of glycolysis, obtained in centrifuged extracts of brain in the absence of added phosphate donors, was due to a removal of the excess of ATP-ase[2] with the particulate matter. Only under these conditions are the activities of hexokinase and ATP-ase, the enzymes mainly responsible for phosphorylation and dephosphorylation of the sugar intermediates, kept in step. In the uncentrifuged homogenate, on the other hand, the ATP-ase is so far in excess that the glycolysis rapidly goes down to very low values, owing to the irreversible dephosphorylation of ATP. This lack of balance can be overcome either by adding large amounts of phosphate donors (HDP, phosphocreatine) or by repeated additions of ATP itself.

Recently, it has been shown that the situation in tumor homogenates and extracts is somewhat different (3); on the one hand, most of the ATP-ase remains in solution when the particulate matter is centrifuged out. On the other hand, the dissolved ATP-ase of tumor is strongly inhibited by some higher members of the narcotic series, while the other glycolytic enzymes remain unaffected. This peculiarity can again be used to produce a very high and steady glycolysis in homogenates and

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[2] Abbreviations: ATP = adenosinetriphosphate; ATP-ase = enzyme splitting the first labile group of ATP; pyro P, labile P of ATP; HDP = hexosediphosphate; DPN = diphosphopyridine nucleotide = cozymase.

extracts of tumors in the absence of P donors. With octyl or decyl alcohol or toluene, Q_{La} values (mm.[3] CO_2 driven out by lactic acid/hr. and mg. dry weight of tissues at 38°C.) of 50 to 70 are obtained nearly constant for 60–80 min.[3]

The question arose whether similar effects can be produced in brain homogenate and other tissue homogenates. It is shown in the following, that some of the narcotic substances give at least a partial inhibition of the adsorbed ATP-ase of the brain homogenate and induce in this way a temporary high rate of glycolysis which usually falls off after 40–50 min.

On the other hand, the homogenate of chicken embryo contains only a small excess of ATP-ase over hexokinase. Consequently, glucose alone is glycolyzed quite well, with Q_{La} values ranging from 10 to 25. The ATP-ase is not inhibited but is mostly activated by the narcotic substances in question; it is, however, inhibited by sodium azide. Sodium azide, therefore, can serve to stabilize glycolysis for at least 80 min. Some additional experiments with tumor homogenates and ATP inhibitors are reported.

Methods and Procedures

The methods were the same as in the preceding papers. Lactic acid was determined manometrically by the Warburg technique, using bicarbonate solution, with 95% N_2–5% CO_2 in the gas space (1,2,3). Brain tissue was used only as homogenate. Chicken embryos, between 5 and 11 days, were obtained from West Chester Farm.[4] The water content of the embryos was between 92 and 94%. Before homogenization the embryos were frozen at −12°C. because this allows a better extraction of the glycolyzing enzymes (see 5).

The same narcotics were used as in the preceding papers. Moreover, digitonin and some other lipide solvents were tested also.

Results

1. *Brain Homogenate*

Dissolved brain ATP-ase is not only not inhibited but is actually activated by octyl alcohol during the first minutes of incubation (3).

[3] In a recent paper of LePage (4), on the glycolysis of tumor homogenates, most of the experiments were done in the presence of fluoride. Therefore, only the oxidation-reduction step and the phosphorylation of glucose occured. Moreover, so much HDP was added, that 75% of the maximum amount of lactic acid was produced even in the absence of glucose (see l.c. p. 1014, Table III). In our experiments, only 15 γ P of HDP = 11 mm.[3] CO_2 are added to prime the reactions.

[4] George F. Shaw, West Chester, Pa.

With adsorbed ATP-ase no effect was found in experiments of short duration. If the incubation is prolonged, and if sugar is present, which in itself diminishes the activity of the ATP-ase by transphosphorylation, a slight inhibition occurs, somewhat more with decyl alcohol then octyl alcohol. Simultaneously, the transphosphorylation to glucose is increased (Table I).

TABLE I

Inhibition of ATP-ase of Brain Homogenate by Higher Alcohols
(0.3 cc. homogenate in 1 cc. total volume, temperature 38°C.)

No.	Time[a]	Addition		Inorganic P split off	Pyro P present	Δ 7′ P	ATP-ase inhibition
		Glucose	ATP-ase inhibitor				
	min.			γ		γ	*per cent*
518	30+0	–			136		
	30+6			58.7		–4.5	
	30+6	–	Decyl alcohol	58.7			0
	30+6	+		43.7		–20.4	
	30+6	+	Decyl alcohol	32.4		–27.4	26
590	0	–			133.6		
	6	–		64.4		–2	
	6	–	Octyl alcohol	70.1			0
	6	+		40.1		–28	
	6	+	Octyl alcohol	33.8		–37	15
	6	+	Decyl alcohol	31.6		–37	21
590a	30+6	–		55.1		–6	
	30+6	–	Octyl alcohol	55.1			0
	30+6	+		42.6		–20	
	30+6	+	Octyl alcohol	33.1		–27	22
	30+6	+	Decyl alcohol	30.4		–25	29

[a] 30 + means incubation of the enzyme with the inhibitor for 30 min. at 38°C. before adding ATP.

In this way a high rate of glycolysis can be obtained (see Figs. 1 and 2 for the effect of octyl alcohol, decyl alcohol and toluene). Because the inhibition of ATP-ase is only about 30%, the effect is not maintained for longer than 40 min. However, by adjusting the amount of homogenate, one can find conditions where the rate is constant or rises during 60–80 min. (see Fig. 1, Curve G + D (2/3)). The Q_{La} values 40–50 correspond to those formerly found in centrifuged extracts under favorable

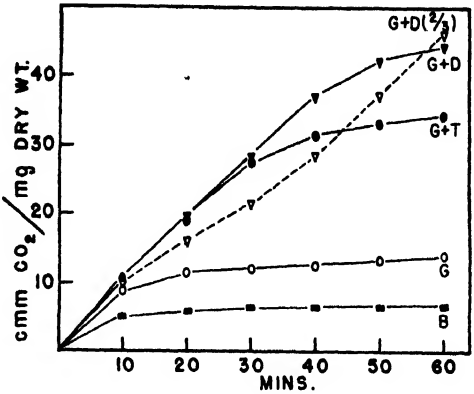

FIG. 1. Glycolysis of complete brain homogenate. In main compartment 0.3 cc. homogenate (10 mg. dry weight). 0.1 cc. $M/10$ phosphate, 0.1 cc. 1.3% $NaHCO_3$, HDP with 15 γ P. Total volume, 1 cc. Side arm: 0.1 cc. ATP with 60 γ pyro P, 0.15 cc. cozymase = 0.6 mg. DPN, 0.05 cc. $NaHCO_3$ 1.3%, tipped in at 0 min.

B, blank without glucose; G, 4 mg. glucose; G + T, glucose + toluene; G + D, glucose + decyl alcohol. Dotted line, G+D (2/3), glucose + decyl alcohol, but only 0.2 cc. of homogenate.

conditions. Smaller increases of glycolysis are obtained with saturated lauryl alcohol, 0.1% digitonin, and 0.2% sodium azide. Some such experiments are reproduced in Table II.

2. *Homogenate of Chicken Embryo*

In the former work on glycolysis of embryonic extracts (5), stress was laid on the oxidation-reduction step and on the transformation of HDP into lactic acid. After recent studies had revealed several conditions necessary for the metabolism of free sugar in the absence of larger amounts of phosphate donors, we applied this knowledge to the homogenate of chicken embryo.

The ATP-ase of this homogenate is almost completely soluble: 75% of the total ATP-ase of the homogenate remains in the extract

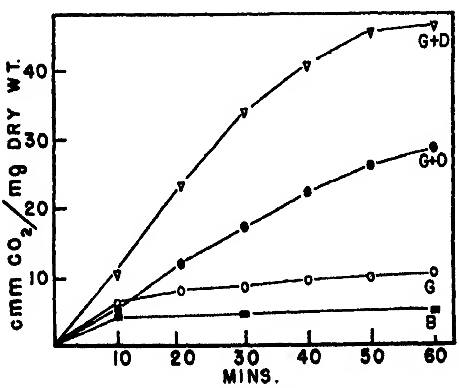

FIG. 2. Glycolysis of brain homogenate. Mixture like that of Fig. 1. B, blank without glucose; G, glucose alone; G + O, glucose + octyl aclohol; G+D, glucose +decyl alcohol.

TABLE II

Q_{La} Values with Glucose, of Brain Homogenate with Various ATP-ase Inhibitors Calculated from 40 min. 38°C.
(Blank value without glucose subtracted.)

No.	Inhibitors used	Conc.	Q_{La}
516	—		6.2
	Octyl alcohol	Satd.	36.8
	Decyl alcohol	Satd.	54.7
	Lauryl alcohol	Satd.	10.5
535	—		11.7
	Decyl alcohol	Satd.	42.2
	Digitonin	0.1%	18.6
	Sodium azide	0.3%	16.2
536	—		10.5
	Decyl alcohol	Satd.	45.2
	Toluene	Satd.	37.2
	Digitonin	0.12%	16.5
	Sodium azide	0.2%	19.0

after centrifugation. This dissolved ATP-ase is activated by octyl alcohol (30–40% for incubation time of 8 min.), quite similarly to the dissolved ATP-ase of brain. It is weakly inhibited by digitonin (0.1%) but more so by 2×10^{-2} *M* sodium azide (35–55%). The Q_P values of ATP-ase are 30–45 in the homogenate. If there were an excess of ATP-ase over hexokinase, only azide could be expected to raise or stabilize the glycolytic rate of free sugar. This was actually the case. While octyl alcohol inhibited the glycolysis increasingly, probably by activation of the ATP-ase, azide could keep it in a steady state when it would otherwise slowly decline. In some experiments the decline during the first 20 min. was very strong and the azide effect pronounced. In such an experiment the following mm.$^3CO_2$ were developed for the time of 20–50 min after the start of the experiment and after subtracting the blank value:

	mm.$^3CO_2$
Glucose alone	25
Glucose with sat. octyl alcohol	5
Glucose with 2×10^{-3} *M* digitonin (0.25%)	56
Glucose with 2×10^{-2} *M* azide	115

In most of the experiments, however, especially if 1×10^{-2} *M* pyruvate was present, glycolysis remained nearly constant more than an hour without azide, with Q_{La} between 10 and 25. If the activity was relatively low, the addition of crystallized yeast hexokinase[5] gave a

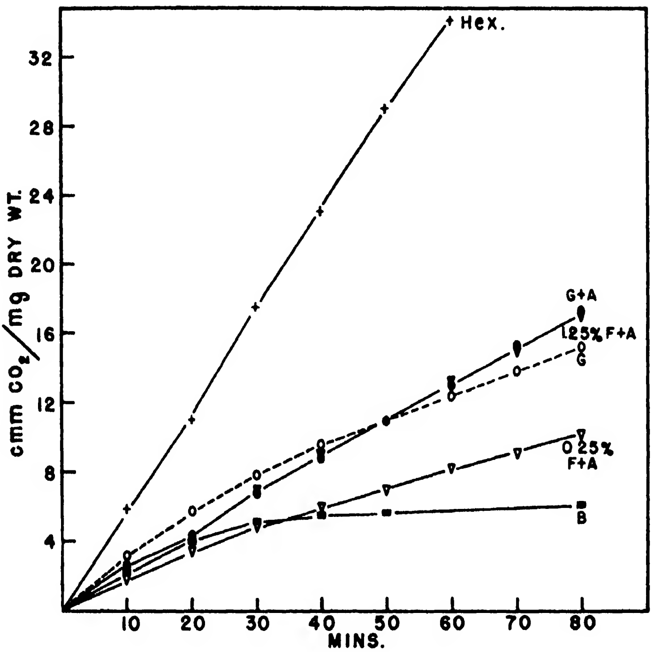

FIG. 3. Glycolysis of homogenate of chicken embryo (587). 7 day old embryo. Main compartment 0.5 cc. homogenate (12 mg. d. w.), 0.1 cc. *M*/10 phosphate, 0.1 cc. *M*/10 pyruvate, 0.1 cc. 1.5% $KHCO_3$, 0.05 cc. *M*/10 glutathione, HDP with 15 γ P filled to 1.35 cc. with 1.15% KCl. Side arm, 0.15 cc. ATP with 90 γ pyro P, 0.1 cc. DPN (0.4 mg.), 0.05 cc. 1.5% $KHCO_3$, tipped in at 0 min.

B, blank without glucose. Dotted line, G glucose alone (0.25%); G + A •—• = 0.25% glucose + 2.5×10^{-2} *M* sodium azide, 1.25% F + A ▼—▼ = 1.25% fructose + 2.5×10^{-2} *M* sodium azide (lower values of the same curve); 0.25% F + A = 0.25% fructose + 2.5×10^{-2} *M* sodium azide; Hex.: glucose 0.25% with addition of 10 γ yeast hexokinase.

considerable increase of activity. In Fig. 3, 10 γ of dry hexokinase powder was used which consists partly of protein and partly of $(NH_4)_2SO_4$. This shows that, at least in these cases, the hexokinase is the enzyme controlling the rate. However, in other experiments, which

[5] We thank Dr. M. Kunitz for a sample of twice crystallized yeast hexokinase.

are summarized in Table III, the Q_{La} value of 25 could be obtained with glucose alone. In this respect it should be remembered that, according to Needham *et al.* (6), the Q_{La} of embryos over 5 days is only about 8 and, with older embryos still lower. Even the lowest rate obtained in the homogenate is still higher than the rate of anaerobic glycolysis of living tissue. It can, moreover, be seen from Table III, that 0.25%

TABLE III

Q_{La} Values in Homogenate and Extract of Chicken Embryo

(For complete mixture see text)

No.	Age embryos in days	D.w. of homog.	Preparation used	Sugar added	Sugar	ATP-ase inhibitor	Deviations from standard mixture	Q_{La}[a] minus blank
		mg.			*per cent*			
583	7	8.5	Homog.	Glucose	0.25		No pyruvate	3
				Glucose	0.25	Sodium azide	No pyruvate	9
				Glucose	1.25	Sodium aizde	No pyruvate	7
				Fructose	0.25	Sodium azide	No pyruvate	3
586	12	18	Homog.	Glucose	0.25			13.2
				Glucose	0.25		No pyruvate	11.1
				Glucose	0.25	Octyl alcohol		11.1
							Yeast hexokinase	34.0[b]
588	10	10	Homog.	Glucose	0.25			25.7
				Glucose	0.25	Sodium azide		22
				Glucose	0.25		30 γ pyro-P instead 90	13.3
				Glucose	0.25	Sodium azide	30 γ pyro-P instead 90	16.1
				Fructose	0.25	Sodium azide	30 γ pyro-P instead 90	9.2
588a	10	10	Extract	Glucose	0.25			14.5
				Glucose	0.25	Sodium azide		14.5
				Fructose	0.25	Sodium azide		8.9

[a] For 60 min. expt.

[b] For 30 min. expt.

fructose in the presence of azide (F + A) gives a much lower rate than 0.25% glucose + azide (G + A). 1.25% fructose, however, gives about the same rate as 0.25% glucose and the points for both are drawn on one curve. Galactose, which is not shown in the figure, gives the same as the blank.

In Table III, the fortified mixture for glycolysis consisted of the following constituents, with 0.3–0.5 cc. homogenate: 0.1 cc. $M/10$ phosphate + 0.1 cc. $M/10$ pyruvate + 0.1 cc. $KHCO_3$ 1.5%, HDP with 15 γ P, 0.05 cc. $M/10$ glutathione, 0.2 cc. 2% sugar, filled to 1.6 cc. with 1.15% KCl and the other additions as stated. The homogenate was made with an isotonic mixture consisting of 67 parts KCl (1.15%), 3 parts $MgCl_2$ and 30 parts $NaHCO_3$ + NH_4HCO_3. In this mixture 2% nicotinamide was dissolved. Cozymase, 0.4 mg. DPN and ATP with 90 γ pyro P were tipped in from the side arm at 0 time.

3. *Additional Observations on Tumor Homogenate*

After the foregoing papers (3) on the glycolysis of malignant tumor homogenates had been published, the analogy between the inhibition of the adsorbed ATP-ase of dried yeast and of the dissolved ATP-ase of malignant tumor had become more and more evident. Therefore, we tested with tumor homogenate those narcotics and lipide solvents which were able to produce the Harden-Young effect in rapidly dried

TABLE IV

Glycolysis of Homogenate of Rat Sarcoma

(8 mg. d.w. of homogenate)

No.	Glucose added	ATP-ase inhibitor	Conc. of inhibitor	Q_{La}^{a}	Q_{La} minus blank
	mg.				
509	—	—		2.7	
	3	—		6.1	3.4
	3	Octyl alcohol	Satd.	28.4	25.7
	3	Benzene	Satd.	27	24.3
	3	Decyl alcohol	Satd.	42	39.2
	3	Digitonin	0.1%	37.8	35.1
	3	Na-desoxy-cholate	0.05%	32.5	29.8
513	—	—		4.1	
	3	—		5.7	1.6
	3	Decyl alcohol	Satd.	52.6	48.5
	3	Lauryl alcohol	Satd.	13.9	9.8
	3	Digitonin	0.03%	60.0	55.9
520	—	—		3.8	
	3	—		4.5	0.7
	3	Octyl alcohol	Satd.	37.0	33.2
	3	Decyl alcohol	Satd.	49.0	45.2
	3	Digitonin	0.08%	41.5	37.7
	3	Digitonin	0.04%	39.0	35.2

Expt. 509, first 10 min., Expt. 513 and 520, first 5 min. not counted.

yeast (7). The following solutions were all able to inhibit dissolved tumor ATP-ase: satd. benzene, satd. chloroform, desoxycholate (0.1–0.2%), and digitonin (0.05–0.25%). Tumor ATP-ase is as sensitive to traces of heavy metals, as had formerly been found for myosine ATP-ase (8). The ATP for these experiments, therefore, must be put through Amberlite resin to remove the traces of mercury resulting from the preparation of ATP. With satd. benzene and chloroform, and with the concentration of digitonin and desoxycholate indicated above, inhibitions of 40–50% are obtained. The same substances are effective in various degrees in eliciting a strong glycolysis by bringing hexokinase and ATP into step. The most suitable of these substances is digitonin, partly because it has no vapor pressure which may interfere with the exact manometric measurement. The Q_{LA} values of 50–60 obtained with digitonin are at least as high as those found formerly with octyl and decyl alcohols (Table IV).

Discussion

From the foregoing experiments in conjunction with those which have been previously published, it seems to follow that the balanced activities of hexokinase and ATP-ase are responsible for maintaining the steady state of glycolysis in homogenates of animal tissues. In contrast to yeast, the hexokinase is the more sensitive enzyme and is relatively deficient, while ATP-ase is mostly in excess. Checking the latter enzyme brings both enzymes into step. In brain homogenate the easiest way to accomplish this is to centrifuge the homogenate, because 9/10 of the ATP-ase is adsorbed on the particles. However, the principle which is effective in tumor, the inhibition of the ATP-ase by higher members of the narcotic series, can be applied in some degree to the adsorbed ATP-ase of brain.

In homogenates of chicken embryos this method gives no result because the ATP-ase is not inhibited by these narcotics. The inhibition by sodium azide can be used for such a purpose. Moreover, the content of ATP-ase is much smaller than in brain and tumor, giving a Q_P of about 30. Therefore, often a quite stable glycolysis is obtained from glucose alone, especially if pyruvate is added; this can be further stabilized by addition of sodium azide.

The inhibition of the dissolved tumor ATP-ase by higher members of the narcotic series and lipide solvents, shows a striking parallelism to the effect of the same substances on the adsorbed but not on the dis-

solved yeast ATP-ase. In this way we found digitonin to be an especially suitable inhibitor of tumor ATP-ase. In a concentration of 10^{-3} *M*, it evokes a steady glycolysis of tumor homogenate with a Q_{La} of about 50.

Summary

The adsorbed ATP-ase of brain homogenate is inhibited 25–30% by decyl alcohol in the presence of glucose and somewhat less by octyl alcohol. This inhibition allows a high glycolysis with a Q_{La} of about 50 for roughly 40 min.

In chicken embryo homogenate the ATP-ase is not inhibited by these narcotics, but is inhibited about 50% by 0.02 *M* sodium azide. Because the ATP-ase of the chicken homogenate is not very much in excess over hexokinase, glycolysis with glucose alone, especially in the presence of pyruvate, is relatively stable. Addition of sodium azide stabilizes it still more.

The ATP-ase of tumor homogenate is inhibited not only by higher alcohols, but also by other lipide solvents, like digitonin. Digitonin, in a concentration of 2.5×10^{-4} *M*, elicits a steady glycolysis of $Q_{La} = 50$, similarly to decyl and octyl alcohols.

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Candidulin: An Antibiotic from *Aspergillus candidus*

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Introduction

An antibiotic substance having marked activity against acid-fast bacteria has been recovered from the fermentation liquor of an organism[1] identified as a member of the *Aspergillus candidus* group of fungi[2] (1). The properties of the active principle distinguish it from antibiotic substances hitherto described, and the name "Candidulin" is therefore proposed for it.

Experimental

Production

The mold was tested for elaboration of the antibiotic by the agar streak method on a variety of substrates, using *Mycobacterium ranae* as the indicator organism. The best activity, as determined by the zone of inhibition of *M. ranae*, was obtained in a medium containing glucose, glycerol, KH_2PO_4, $NaNO_3$, and asparagine. Media containing such complex materials as corn steep, yeast extract, and soybean flour were completely unproductive.

Active fermentation liquor was first produced in a neutral medium consisting of 0.5% glucose, 1.5% glycerol, 0.2% asparagine, 0.1% $NaNO_3$, 0.05% KH_2PO_4, and 2% suspended agar. It was later found that, if asparagine was omitted from the broth, agar was no longer required for the elaboration of the antibiotic. In either case, metabolism solutions assaying 64 dilution units per ml. (tested in Kirchner's medium against *M. ranae*) was regularly obtained under stationary conditions after 7 days of incubation at 25°C. in 500 ml. Erlenmeyer flasks, 100 ml. per flask.

[1] A colony of the organism appeared on a pour plate of the vomitus of "Ruppel's" vulture. This material was examined at the suggestion of Dr. David Rutstein, Harvard University, and supplied through the courtesy of the Bronx Zoo, New York, N. Y The mold was not typical of the flora of the vomitus, since only one colony appeared on a single plate of a dozen or more poured. The predominant organisms present were yeasts.

[2] Dr. Kenneth B. Raper of the Northern Regional Research Laboratories has kindly examined the mold and concurs with this identification.

Stability

At pH 2, 5, and 7, the activity of metabolism liquor was unimpaired after 1 hr. at room temperature or 10 min. at 100°C. At pH 9 it was stable for at least 1 hr. at room temperature, but reduced after 10 min. at 100°C. At pH 11, the activity was reduced after 5 min. and destroyed after 1 hr. at room temperature or 10 min. at 100°C.

Extraction

One volume of ether completely removed the activity from metabolism liquor at pH 2, 7, or 10.7, suggesting, therefore, a neutral molecule. Two or 3 extractions with 1/10 volumes of chloroform sufficed to remove all the activity from metabolism solutions at their prevailing pH (7.5–7.7).

Purification and Crystallization

Chloroform extracts so obtained were washed with one 1/10 volume of water, dried with Drierite and distilled *in vacuo*, leaving an oily, viscous residue containing the active principle.

A degree of purification of this oil could be achieved by chromatographic methods. For example, a chloroform solution percolated through a column of $MgSO_4$ allowed free passage of the active principle while retaining certain of the colored imputities. On the other hand, when percolated through a column of activated alumina, the antibiotic was firmly adsorbed, whereas certain of the impurities passed through freely. Other impurities could be removed by development with a solution of 10% ethanol in chloroform. Under these conditions, slow movement of the active principle took place so that, upon the addition of 7–9 column volumes (column = 9″ × 3/4″), the antibiotic appeared in the eluate. Nevertheless, chromatography was of limited preparative value because there was no simple way of following the movement of the active principle, and because good separation from various other impurities was not readily achieved. It was, however, useful in working up very crude materials, such as mother liquors, from which further crystalline material could not otherwise be obtained.

Routine preparation of crystalline candidulin was accomplished by digestion of the chloroform-free, dry residue with several portions of boiling *n*-hexane, from which the active principle separated out, on standing and cooling, in crystalline form. The crude, yellowish crystals were repeatedly recrystallized from *n*-hexane until white and free of viscous impurities. The purer the antibiotic became, the more readily it separated out from hexane as a voluminous mass of long, white, glistening, needle-like particles. The yield of purified material was about 5 mg./l. of medium.

Physical and Chemical Properties

Repeatedly recrystallized candidulin melted at 88–89°C. (hot stage, uncorr.) and was optically active, $[\alpha]_D^{24} = +15° \pm 2°$ (1% in

chloroform). Qualitative tests indicated nitrogen to be present and halogen and sulfur absent.

Analysis:

	C	H	N (Dumas)
Found	63.47	7.3	6.6
	63.53	6.9	
Calculated for $C_{11}H_{15}NO_3$, M. W. 209.13			
	63.1	7.2	6.7

The molecular weight, determined by the osmometric method (2) in water, was 232, indicating that the true molecular weight corresponds to the empirical formula given above.

The following crystallographic properties characterized the substance:

Crystal habit: Extremely thin elongated plates with longitudinal striations.

Refractive indices: N_x and N_y = 1.525 ± 0.003.

Optic sign: Positive.

Optic axial angles: 2E 113°, 2V 67°

The material contained no titratable acid or basic groups. After standing at pH 10.9 and back-titrating, a fairly well-defined inflection appeared, corresponding to a pK of 9.4. The resulting solution was devoid of antibiotic activity and gave no ninhydrin or $FeCl_3$ tests.

The approximate solubility (mg./ml.) of candidulin at room temperature was determined as follows: methanol, ethanol, acetone, ether and chloroform, > 6; benzene, 5; carbon tetrachloride, 4; *n*-hexane, 0.075; *n*-hexane at b.p., about 1; water, 5 (slowly); 1 *N* HCl and 5% Na_2CO_3, insoluble or slowly soluble as in water; dilute NaOH, very rapidly soluble, presumably due to hydrolysis.

Candidulin showed no ultraviolet absorption. Examination of its infrared spectrum, using a Nujol mull, suggested the following structures to be present: methyl, internal unsaturation, carbonyl, amine and possibly hydroxyl. No absorption corresponding to the phenyl radical was present.

The $FeCl_3$, fuchsin aldehyde and ninhydrin tests were negative. No solid products were obtained by treatment with 2,4-dinitrophenylhydrazine, 3,5-dinitrobenzoylchloride and other acylating reagents, xanthydrol, and HgO.

Treatment with bromine in CCl_4 afforded a biologically inactive, crystalline, halogen-containing product in low yield. This was obtained as follows: 34 mg. of candidulin was dissolved in a minimal quantity of CCl_4 and treated, dropwise, at room temperature, with a solution of 4 ml. of bromine in 100 ml. of CCl_4 until the color of bromine persisted. The solvent was distilled *in vacuo* and the residue crystallized from ethanol-water or methyl ethyl ketone, yielding 2.6 mg. of a white crystalline product, m.p. 143-147°C. (decompn.). The following refractive indices of the columnar crystals were obtained:

$$N_1 \text{ parallel length} = 1.665 \pm 0.003,$$
$$N_2 \text{ perpendicular length} = 1.610 \pm 0.003.$$

TABLE I

Antimicrobial Spectrum of Candidulin

Organism	Inhib. conc.[a] γ/ml.
In Trypticase-Soy Broth	
S. aureus (152)	320
S. aureus (II)	320
Sarcina lutea	320
Strep. hemolyticus (C203)	160
B. polymyxa (A2)	160
B. subtilis (398)	10
Ps. aeruginosa	640
E. coli (MacLeod)	160
B. abortus (19)	20
K. pneumoniae (BE)	10
S. griseus	320
A. fumigatus	80
P. puberulum	40
Mycobacterium (607)	1.25
M. ranae	2.5
M. smegmatis	2.5
In Kirchner's Broth[b]	
M. ranae	0.4
M. tuberculosis (H37)	0.1
M. tuberculosis (D4)	0.06

[a] Observations made after an incubation period of 24 hr. at 37°C. with these exceptions: *S. griseus, A. fumigatus* and *P. puberulum,* 72 hr.; the saprophytic mycobacteria, 48 hr.; and the tubercle bacilli, 7 days.

[b] Containing 0.05% Tween 80.

Biological Properites

Antimicrobial Spectrum

Representative organisms were tested for their response to candidulin by serial two-fold titration in broth. The results are shown in Table I. It is apparent that the *Mycobacteria* were particularly sensitive to the antibiotic.

Lethal Toxicity for Mice

A limited number of mice (1 or 2 per dose) were injected subcutaneously with an aqueous 0.5% solution of candidulin in doses of from 25 to 300 mg./kg. The results suggested that the LD_{50} was in the neighborhood of 250 mg./kg.

Therapeutic Test

Groups of 5 mice infected intravenously with *M. tuberculosis*, strain D4, were treated subcutaneously for a maximum of 12 days with daily doses of 25, 50, 100, and 200 mg./kg. beginning on the day of infection. The treated mice survived no longer than the control mice, and of those examined *post mortem*, all showed characteristic lesions of tuberculosis. It was apparent, therefore, that candidulin exerted no effect on the course of this experimental infection.

Miscellaneous

A limited investigation was undertaken to determine the possible fate of candidulin in the mouse when administered in a single dose orally (200 mg./kg.), subcutaneously, intraperitoneally or intravenously (each at 100 mg./kg.). In no case was it possible to demonstrate antibiotic activity in the blood or urine when tested at intervals 5 min. to 6 hr. after administration. The stomach contents of the mouse which received the oral dose showed appreciable activity 6 hr. after administration. Some activity was also present after 6 hr. at the subcutaneous site of injection of the mouse receiving this treatment. Preliminary observations indicated that incubation of candidulin with mouse liver, kidney, plasma, and washed erythrocytes did not affect activity very much, whereas whole blood had a marked neutralizing effect.

Acknowledgment

Grateful acknowledgment is made to the following, all of these Laboratories: Dr. P. H. Bell and Miss K. S. Howard for the molecular weight determination and titration; Mr. R. J. Francel for the infrared examination; Dr. A. F. Kirkpatrick for the crystallography; Dr. J. A. Kuck and staff for the microanalysis; and Dr. H. J. White and staff for the chemotherapeutic test.

Summary

A crystalline substance having marked antibiotic activity against acid-fast bacteria was recovered from the fermentation liquor of a strain of *Aspergillus candidus*. The production, isolation, physical, chemical and biological properties of the active prinicple, for which the name "Candidulin" is proposed, are described. The material failed to influence the course of experimental mouse tuberculosis.

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Destruction of Influenza A Virus Infectivity by Formaldehyde [1,2]

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Introduction

The destruction of the infectivity of influenza virus is of considerable practical importance because influenza vaccines are prepared by inactivating virus. Studies on the kinetics of the inactivation of influenza virus by heat (1) and by the action of urea (2) have been reported previously. The present communication deals with the inactivation of influenza in the presence of formaldehyde.

Materials and Methods

PR8 influenza A virus, the history of which was described previously (2), was used in the present investigation. Preparations A through J represent the 8th to 11th and 14th to 19th chicken embryo passages in Pittsburgh, respectively. Each virus preparaiton consisted of allantoic fluid from infected chicken embryos. Potassium phosphate buffers of ionic strength 0.2 at pH values of 4.7, 5.6, 5.8, 7.0 and 8.0, and sodium acetate-acetic acid buffers of ionic strength 0.2 at pH values of 5.0, 5.2 and 5.4, were used in these investigations. The formaldehyde was of reagent grade.

The general method of study was to dilute 0.1 ml. of virus preparation to 10 ml. with buffer and formaldehyde. In most of the studies, the final formaldehyde concentration was 1×10^{-4} g./ml., but the range was from 0 to 2×10^{-4} g./ml. The final virus concentrations were 1% of those in the original allantoic fluid preparations. The solution containing virus and formaldehyde was then heated in a water bath held at some particular temperature, and samples were removed periodically for analysis. Fifty per cent chicken embryo infectivity end-points were determined in the manner described previously (1,2). It was found that the inactivation in the presence of formaldehyde was probably a first order reaction with respect to virus concentration. Accordingly, first order reaction velocity constants and their standard errors were computed in the manner described previously (2).

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[1] Aided by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[2] Contribution No. 4-p-49, of the Department of Physics of the University of Pittsburgh.

Experimental Results

In Table I are presented specific reaction velocity constants and their standard errors for the destruction of infectivity of PR8 influenza A virus in the presence of formaldehyde. Ten different virus preparations were used. The effects of three variables, temperature, formaldehyde concentration and pH, were investigated. The temperature range studied was 25–40°C.; the formaldehyde concentration range was 0–2 × 10^{-4} g./ml.; and the pH interval was 4.7–8.0.

The reproducibility of rate constants for destruction of infectivity in formaldehyde at 10^{-4} g./ml. for different virus preparations can be ascertained by examining certain of the values presented in Table I. The standard error for the difference between two values is the square root of the sum of the squares of the standard errors of two values. In only one case, that involving preparations F and I at 25°C., pH 5.6, did the difference between rate constants exceed twice the standard error of difference. On the basis of these results, it is reasonable to conclude that rate constants are reproducible, within experimental error, from preparation to preparation.

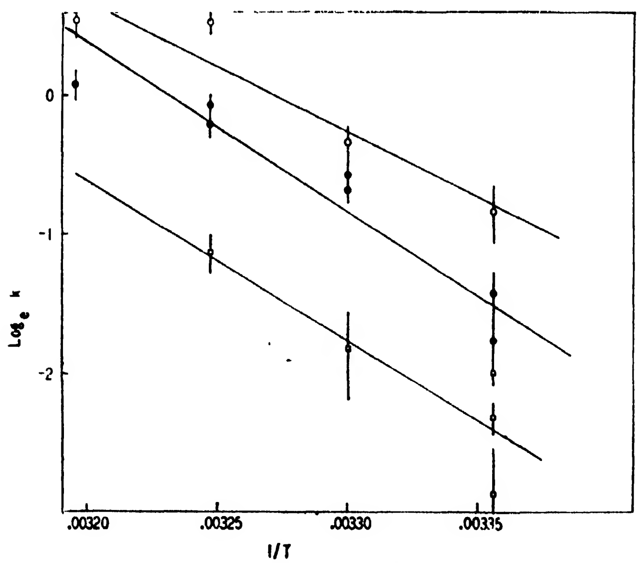

Fig. 1. Natural logs of reaction velocity constants for the destruction of PR8 influenza A virus infectivity plotted as ordinates against reciprocals of absolute temperature. Top graph—pH8; middle graph—pH 7; bottom graph—pH 5.6. Formaldehyde concentration was 1 part/10,000.

TABLE I

The Destruction of Influenza Virus Infectivity in the Presence of Formaldehyde

pH	Formaldehyde concentration g./ml. $\times 10^4$	Temperature °C.	k min^{-1}	SE k	Preparation
8.0	0.5	25	0.25	0.02	D
8.0	1.0	25	0.44	0.09	D
8.0	1.0	30	2.21[a]	0.39	C
8.0	1.0	30	0.71	0.09	D
8.0	1.0	35	1.77	0.39	C
8.0	1.0	35	1.70	0.14	D
8.0	1.0	40	1.72	0.20	D
8.0	2.0	25	0.74	0.07	D
7.0	0.0	25	0	—	J
7.0	0.5	30	0.34	0.05	A
7.0	1	25	0.24	0.04	E
7.0	1	25	0.17	0.04	J
7.0	1	30	0.51	0.05	A
7.0	1	30	0.56	0.06	E
7.0	1	35	0.94	0.09	A
7.0	1	35	0.82	0.08	E
7.0	1	40	1.09	0.12	E
7.0	2	30	1.15	0.25	A
5.8	1	25	0.08	0.02	G
5.6	0.0	25	0.11	0.03	I
5.6	0.5	25	0.06	0.01	D
5.6	1	25	0.10	0.01	D
5.6	1	25	0.06	0.02	F
5.6	1	25	0.13	0.01	I
5.6	1	30	0.16	0.05	C
5.6	1	35	0.32	0.05	C
5.6	2	25	0.17	0.02	D
5.4	1	25	0.06	0.02	G
5.2	1	25	0.20	0.04	G
5.0	0	25	0.41	0.09	H
5.0	1	25	0.49	0.10	H
5.0	1	25	0.43	0.06	G
4.7	0	25	0.65	0.13	J
4.7	1	25	0.65	0.11	J
Unbuffered	1	25	0.13	0.02	B
Unbuffered	1	30	0.46	0.07	B
Unbuffered	1	35	0.78	0.14	B
Unbuffered	1	40	2.76	0.46	B

[a] This value was not used in any computations because it is obviously inconsistent with the pattern established by remaining data.

Some of the data of Table I are grouped in Fig. 1 in a manner to show the way in which rate constants vary with temperature. Natural logarithms of rate constants are plotted against reciprocals of absolute temperature, according to the Arrhenius equation, for studies carried out at pH 5.6, 7.0 and 8.0. It can be seen that the data fall reasonably well upon straight lines. Energies of activation, and entropies of activation, can be computed in the manner described previously (1,2.) The energies of activation were 23,000, 24,000 and 19,000 cal./mole, and the entropies of activation were 11, 18 and 0 cal./mole deg., respectively for the reactions at pH 5.6, 7.0 and 8.0. The differences between these energies of activation and entropies of activation at the different pH values are of doubtful significance, for each figure is subject to considerable experimental error. The important thing is that, for the destruction

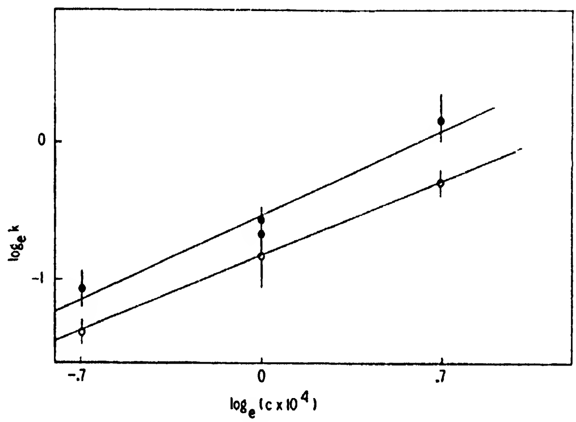

FIG 2. Natural logs of reaction velocity constants for the destruction of PR8 influenza A virus infectivity plotted as ordinates against natural logs of formaldehyde concentration expressed in terms of parts/10,000. Upper graph—pH 7, 30°C ; lower graph—pH 8, 25°C

of PR8 influenza A virus infectivity in formaldehyde solutions at a concentration of 10^{-4} g./ml., the energy of activation is about 20,000 cal./mole and the entropy of activation is approximately 10 cal./mole deg. at pH values between 5.6 and 8.0.

In Fig. 2, natural logs of specific reaction rates are plotted against natural logs of formaldehyde concentrations for studies carried out at 30°C., pH 7.0, and at 25°C., pH 8.0. In both cases, the data fell on straight lines with slopes of approximately 0.8. This means that the

reaction velocity constants are proportional to the formaldehyde concentration raised to approximately the 0.8 power.

The effect of variation of pH upon the specific reaction velocity constants is shown in Figs. 3 and 4. In Fig. 3, logs of specific reaction rates

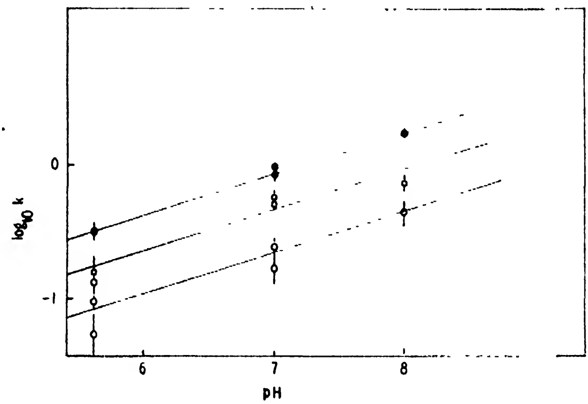

FIG. 3. Logs of specific reaction velocity constants plotted as ordinates against pH. Upper graph—35°C.; middle graph—30°C.; lower graph—25°C. Formaldehyde concentration was 1 part /10,000.

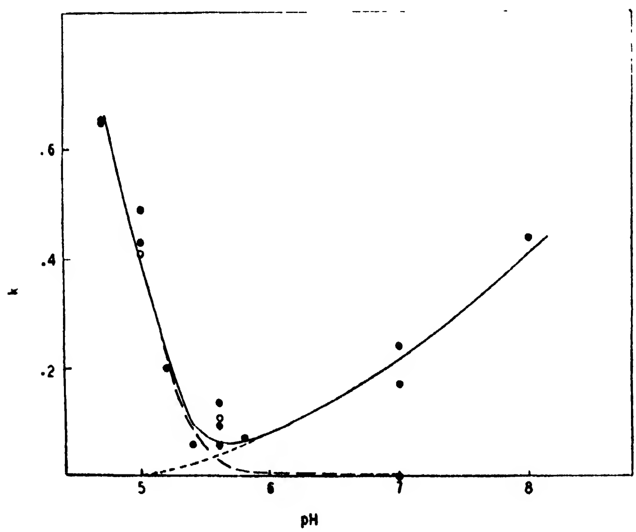

Fig. 4. Reaction velocity constants plotted as ordinates against pH for range from 4.7 to 8.0. Broken line and open circles represent rates in absence of formaldehyde. Dotted line and closed circles represent rates in presence of 1 part formaldehyde/ 10,000. Solid line represents sum of the other two.

are plotted against pH, in the range 5.6–8.0, for formaldehyde concentrations of 10^{-4} g./ml. at 25, 30 and 35°C. The slopes of the three graphs are all approximately 0.3. This would indicate that the specific reaction rate for the destruction of influenza A virus infectivity in formaldehyde in media alkaline to the isoelectric point is proportional to the reciprocal of the hydrogen ion activity raised to the 0.3 power. In Fig. 4, reaction velocity constants at 25°C. are plotted against pH for studies carried out in the absence of formaldehyde and in 10^{-4} g. formaldehyde/ml. The pH range covered was from 4.7 to 8.0. It can be observed that the rate of destruction of infectivity in the presence of formaldehyde has a minimum value under the conditions of this experiment at a pH value of about 5.6, and also that the rate of destruction of infectivity in media more acid than pH 5.6 is the same in the presence of formaldehyde as in the absence of formaldehyde.

Discussion

The kinetics of the reaction have been investigated. According to most current concepts of reaction kinetics, the way in which an agent can accelerate a reaction is to combine with the reactant to form an intermediate product. There is chemical evidence (3,4) that formaldehyde is capable of reaction with amine, amide, indole and guanidine residues. If the enhanced reaction rate observed in the presence of formaldehyde is the result of the formation of an intermediate compound with formaldehyde, the data of the present study indicate something of the possible nature of that reaction.

It is a necessary deduction from the above postulate, namely, that formaldehyde speeds up the destruction of virus activity by the formation of an intermediate compound which then loses infectivity, that the rate of loss of infectivity should be proportional to the fraction of the virus particles which exist as virus-formaldehyde complex at any one instant. According to the mass action law, this fraction should be equal to the product of the equilibrium constant for complex formation, and the formaldehyde concentration raised to the nth power, where n is the number of formaldehyde molecules which react with one virus particle to form the complex. It follows that, if these postulates apply, the rate of the reaction should be proportional to the formaldehyde concentration raised to the nth power. The data of Fig. 2 show that the rate of destruction is proportional to the formaldehyde concentration raised to the 0.8 power. If it is assumed that this figure does not differ

significantly from 1, then these data tend to show that the reaction between virus and formaldehyde involves only a single formaldehyde molecule for each reactive group on a virus particle. Thus, one might say that a particular group, or, perhaps, one of several particular groups, on a virus particle can react with one molecule of formaldehyde to form a complex which loses infectivity more rapidly than normal virus.

The chemical groups which might conceivably react with formaldehyde exist in nonionized form in alkaline solutions but are capable of capturing protons to form positively charged groups in acidic solutions. These groups seem to react in the nonionized form (4) with formaldehyde. If this is so, one should expect the equilibrium between virus-formaldehyde complex and unaltered virus to depend upon the pH of the medium; the higher the pH, the higher the fraction in the complex state and, consequently, the higher the rate of the desturction of influenza virus activity. The data of Fig. 3 show that this prediction holds over the pH range 5.6–8.0. However, the data of Fig. 4 show that the reaction has a minimum rate at approximately pH 5.6. The isoelectric point of PR8 influenza A virus has been shown to be at pH 5.3 (5). It is tempting to dismiss the approximate coincidence between the isoelectric point and the pH value of minimum destruction rate by the generalization that many biologically active materials are most stable at their isoelectric points. However, it was shown (1) that, in the absence of formaldehyde, the destruction of activity proceeds at a minimum rate at about pH 8 and at faster rates in media with lower pH values. This knowledge suggested the possibility that two reactions are involved in the destruction of infectivity in the presence of formaldehyde. The first of them involves reaction with formaldehyde to form a complex which then loses infectivity. The second does not involve reaction with formaldehyde, but is simply the thermal inactivation of the virus. Accordingly, it can be assumed that, at pH 8 in the presence of formaldehyde, the first reaction proceeds at an appreciable rate at 25°C. and the second reaction proceeds at negligible rate. As pH is lowered, the rate of the first reaction decreases and the rate of the second reaction increases. The results of the present study can thus be accounted for by assuming that, at pH 5.6, or thereabouts, the rate of the second reaction becomes greater than that of the first, and that, at pH values below this, the variation of rate with pH depends upon the variation of thermal inactivation with pH. If this assumption is correct,

then the following should be observed: (a) the rate of reaction at pH values substantially below 5.6 should be the same in the absence of formaldehyde and in the presence of formaldehyde; (b) at pH values substantially above 5.6, the reaction rate should be considerably greater in the presence of formaldehyde than in the absence of formaldehyde. Data shown in Fig. 4 confirm this prediction, for the rates of inactivation at pH 4.7 and pH 5.0 are the same in the presence of 1 part/10,000 of formaldehyde and in the absence of formaldehyde, but at pH 7 the reaction in the presence of formaldehyde proceeds at a measurable rate while that in the absence of formaldehyde proceeds too slowly to be measured. The possiblity also exists that, at some pH values much higher than 8, the recation which accounts for the thermal inactivation at high pH values (1) might proceed faster than that involving formaldehyde.

SUMMARY

1. The inactivation of PR8 influenza A virus in the presence of formaldehyde was investigated.

2. The reaction is of the first order with respect to virus.

3. The energy of activation is approximately 20,000 cal./mole and the entropy of activation is approximately 10 cal./mole deg.

4. At pH values between 7.0 and 8.0, the rate of inactivation is proportional to the formaldehyde concentration raised to approximately the 0.8 power, but at pH values below 5.6, the rate is independent of formaldehyde concentration.

5. Between pH 5.6 and 8.0, the rate of inactivation decreases as pH decreases. Below pH 5.6, the rate of reaction increases as pH decreases. The reaction below pH 5.6 was shown to be the same as the normal heat inactivation of the virus.

6. The data are consistent with the interpretation that, in the pH range studied, two parallel reactions contribute to the loss of infectivity. One involves the reaction of one molecule of formaldehyde with the nonionized form of an ionizable group on a virus particle to form a complex which then loses activity readily. This reaction predominates at pH values above 5.6. The other reaction is the normal thermal inactivation process which does not involve reaction with formaldehyde. This process predominates at pH values below pH 5.6.

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An Unknown Effect of Amino Acids. II. Interaction of Nitrogenous Polycarboxylic Acids (N-Substituted Amino Acids) and Insoluble Metal Sulfides and Mercaptides

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Introduction

Amino acids and related substances in neutral or weakly alkaline solution have been shown (1) to prevent precipitation of the insoluble sulfides of many metals, and also to redissolve freshly precipitated sulfides. The same was found true for mercaptides. The importance of this phenomenon is due to the fact that it applies to substances widely distributed in nature: protein degradation products, biometals, and thiol compounds occurring during metabolism.

A possible explanation of the phenomenon may involve formation of a complex between metal and amino acid of sufficient stability to prevent precipitation with H_2S or to effect a double decomposition of the precipitated metal sulfide on interaction with excess amino acid in weakly alkaline solution.

Some understanding of these reactions might be gained by a study of the behavior of certain nitrogenous polycarboxylic acids such as ethylenediaminetetraacetic acid $(COOH \cdot CH_2)_2{:}N \cdot CH_2 \cdot CH_2 \cdot N{:}(CH_2 \cdot COOH)_2$ and trimethylaminetricarboxylic acid $(COOH \cdot CH_2)_2{:}N \cdot CH_2 \cdot COOH$. These substances have not been found in nature—at least as yet—but their great tendency to complex formation was established by Ender, Brintziger, Pfeiffer, Schwarzenbach, and others (2). They may be regarded as *N*-substituted amino acids or glycine derivatives.

The results tabulated below show that solutions of both compounds at a pH of approximately 8 prevent the precipitation of the sulfides of Zn, Co, Ni, Mn, Fe^{++}, Fe^{+++}, VO^{++}. In this respect they resemble

natural amino acids. They also prevent sulfide precipitation of lead, the biological significance of which has recently been pointed out (3). On the other hand, they do not prevent precipitation of CuS under conditions in which ordinary amino acids were found effective and their ability to redissolve CoS is negligibly small. The sulfides of Ag, Cd, Hg, Tl, and Bi are precipitated in the presence of both reagents. In spite of the great tendency to complex formation of these nitrogenous polycarboxylic acids, and although Ag, Cu, Cd, Hg, and Bi[1] are known to form complexes, these criteria do not allow a prediction of the behavior of these systems toward H_2S or mercaptans. Quite generally, a tendency to complex formation *per se* will not inhibit precipitation as the sulfide. A well-known example applied in inorganic analysis is the resistance of many metal complexes toward alkali hydroxides. Some metals, which cannot be precipitated as hydroxides in the presence of glycerin, tartrates or citrates, can still be precipitated as sulfides, *e. g.*, Fe, Cu, Bi, Co, Ni.

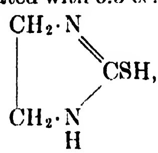
In this connection, reference may be made to the possible feasibility of using nitrogenous polycarboxylic acids in the same way as polyphosphates (5) and nucleinates (6) when the effectiveness of the bioelements is to be assured in enzyme systems, in metalloproteins or otherwise, and deionization must be retained to guard against inhibition by thiol compounds.

Experimental

The exact procedure and all necessary precautions as well as the advantages of Na_2S as precipitating agent have been fully described in a previous publication (1).

The trimethylamine tricarboxylic acid (Eastman-Kodak) was purified by solution in alkali and reprecipitation with HCl, or by recrystallization. A molar solution of the disodium salt in water, pH approximately 7, was used. In the case of the ethylenediaminetetraacetic acid, which can be purified in the same way M solutions of the trisodium salt, pH approximately 8.5, have been applied.

In all experiments $M/100$ solutions of the metal salts were precipitated with 0.5 cc. $N/10$ Na_2S or $M/10$ solutions of the Na salt of ethylene isothiourea,



[1] This has been specifically established in the case of Bi triglycolamate (4).

as a convenient mercaptan-like substance, and redissolved by means of 2 cc. of the nitrogenous polycarboxylate solutions. Control experiments were made in all cases. They showed that 1 cc. of $M/100$ metal salt solution was required to obtain a definite ethylene isothiourea precipitate and also for CuS and ZnS. One-tenth cc. proved sufficient for the precipitation of all other metal sulfides. One cc. of $M/10$ Zn salt was used for the Zn ethylene-isothiourea precipitation.

Freshly precipitated CoS could not be redissolved completely, but precipitation could easily be prevented when Na_2S was added to the Co salt in the presence of either polycarboxylate.

The ethylenethiourea was dissolved in an equimolecular quantity of NaOH and a few drops methanol added to facilitate solution.

*Xanthogenates, which were investigated in some cases, were similarly redissolved.

In all cases, identically the same results were obtained with the disodium trimethylaminetricarboxylate and the trisodium ethylenetetraacetate solutions.

Unless the contrary is specifically indicated, all solutions tabulated below remained clear for days.

	Na_2S	Na ethylene-isothiourea	K ethyl xanthogenate
Zn	Clear on warming	Clear	Clear
Ni	Clear	Clear	Clear
Co	Prevents only	Clear	Prevents only
Mn	Clear	Clear	[a]
Fe^{++}	Clear on warming or standing	Clear	[a]
Fe^{+++}	Clear on warming and standing	—	Clear
Pb	Clear on warming for short time only	Clear	Clear
Cu	No effect	Clear	Clear on warming
Tl	No effect	Clear	
Ag	No effect	Clear	
Cd	No effect	Clear	
Hg	No effect	Clear	
Bi	No effect	Clear	
VO^{++}	Clear[b]	Clear	
UO_2^{++}	Clear on standing	Clear	

[a] Lack of a definite precipitate prevented making satisfactory tests with these substances.

[b] The grayish brown precipitate formed on addition of Na_2S to vanadyl sulfate, dissolved without difficulty in both reagents. Whether a true sulfide is involved must be left, however, in abeyance, since a precipitate is also formed when NaOH is added to the blue vanadyl compound. This precipitate too dissolves in both reagents, though with a different color.

Acknowledgment

The authors wish to thank Rohm & Haas Co., Philadelphia, Bersworth Chemical Co., Farmingham, Mass., and Alrose Chemical Co., Providence, R. I., for supplying them with chemicals used in this investigation.

Summary

N-substituted polycarboxylic amino acids are shown to prevent precipitation of sulfides, mercaptides and xanthogenates, and to dissolve freshly precipitated thiol compounds with an effectiveness equal to but not greater than that of ordinary amino acids.

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Biochemical Individuality. III. Genetotrophic Factors in the Etiology of Alcoholism [1]

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Introduction

Recent developments in the field of biochemical genetics (1) make it inescapable that each individual possesses a distinctive hereditary make-up and a metabolism which is distinctive as to its details because of differences in enzymic patterns (2,3). Partial genetic blocks (4,5) somewhere along the metabolic assembly line are probably commonplace in the inheritance of individuals and these may give rise to augmented requirements for specific minerals, vitamins, amino acids, or other nutritional factors. On the basis of these genetic variations, it becomes possible for one individual to suffer from nutritional disease when his diet is wholly adequate from the standpoint of many other individuals.

If attention is centered on the classification of those diseases in which no infective agent appears to be involved, *nutritional* diseases such as pellagra, rickets, scurvy and beri beri, and diseases of *genetic* origin such as hemophilia, Huntington's chorea, *etc.*, constitute highly important groups. So far as we know, however, no one has given attention to the existence of *genetotrophic* (geneto = genetic; trophic = nutritional) diseases—those in which the genetic background and nutritional factors jointly enter into the etiology.

We wish to present the hypothesis that numerous diseases of obscure etiology are genetotrophic in origin. With respect to alcoholism, we have strong evidence which is to be presented in this paper. Other diseases such as allergies, mental diseases, cardiovascular diseases, arthritis, multiple sclerosis, drug addiction and cancer, have not been studied

[1] This research was supported in part by grants from the Research Corporation and the Research Council on Problems of Alcohol, New York.

from this standpoint, but we believe that the facts warrant such a study and that careful consideration should be given to the possibility, and even probability in some cases, that genetotrophic factors are operative.

That these factors enter in an important way into the etiology of a particular disease does not exclude the entrance also of psychogenic or other influences. Even diseases which are primarily due to infective agents may in turn be greatly affected by genetotrophic factors. An individual may, for example, possess a metabolic pattern which is conducive to a specific nutritional deficiency; this deficiency in turn may constitute an invitation to infection even though the available evidence on this point is not impressive (6).

Alcoholism, or compulsive drinking considered as a disease, continues to be one for which neither a practical means of prevention nor a satisfactory treatment has been developed (7). One of us, on the basis of new clarification and insights gained in the field of biochemical genetics and on a careful survey of the pertinent literature, has outlined reasons for suspecting that the extreme appetite for alcohol possessed by compulsive drinkers has a physiological basis closely linked with the inherited metabolic patterns of the individuals afflicted (8). Other specific appetites, *e.g.*, for salt, for calcium, for phosphate, for sugar as in certain diabetics, for fat, for protein, for B vitamins, all have a physiological basis, and there is ample reason for supposing that the appetite for alcohol has also. This hypothesis does not exclude the influence of psychological and social forces in alcoholism, but emphasizes that, in the study of the physiological aspects of acloholism, attention must be directed to individual metabolic patterns as distinguished from the hypothetical pattern common to all individuals.

Studies with experimental animals here reported offer confirmation of this hypothesis and make possible a theory of alcoholism which is in line with all the previously known facts as well as the new findings presented. In addition, these studies strongly suggest the possibility of developing treatments, both prophylactic and therapeutic, for the management of this disease. Such treatments will, in the nature of the case, act in a constructive manner and improve the general health of the patient.

Experimental

Approximately 100 white rats and 30 mice have been placed in individual cages and their alcohol consumption observed. For this purpose young weanling animals have

generally been used. Two drinking bottles have been provided, one containing water and the other 10% alcohol, and the positions of the drinking bottles have been interchanged daily. The consumption of alcohol, water and food was followed daily for many of the animals. However, when they were not under treatment, as much as 3 days sometimes elapsed between readings. The food consumption records have not been analyzed or utilized, partly because of their unreliability due to scattering.

In addition to carrying out the tests with animals on stock diets, two experimental diets have been used. Diet A contained:

Commercial casein	20 parts
Sucrose	60 parts
Vegetable oil (Wesson Oil)	15 parts (fortified to give 60 units Vitamin A and 10 units Vitamin D per cc.)
Dried brewers' yeast (autoclaved)	10 parts
Salt mixture No. 2	5 parts

The yeast was autoclaved at pH 9.0 for 90 min. at 125°C.
Diet B had the same composition except for the addition, in mg./kg. of diet, of:

Thiamine	1
Pyridoxine	1.5
Riboflavin	7.5
Choline	1000
Calcium pantothenate	30
Inositol	30
p-Aminobenzoic acid	30
Folic acid (synthetic *L. casei* factor)	1
Nicotinic acid	10
Biotin	0.1

In the first experiments, animals were used from our original stock of Wistar rats which had been grown in our laboratory over a period of years. These were designated "Strain O." Later, similar rats were obtained from a neighboring laboratory and designated "Strain H." Three strains of mice were used, including (1) a dba strain which was closely inbred using brother-sister matings, (2) a C_3H strain for which, because of breeding difficulties, brother-sister mating had been abandoned, and (3) a strain of white mice purchased on the market.

Results and Discussion

Inspection of the alcohol consumption records shows clearly that each animal tends to exhibit a distinctive pattern of response. These patterns, however, fall into a few general groups, and show a parallel to the diverse drinking patterns of human individuals. One group of rats on a stock diet, exemplified by Fig. 1, consumed relatively large amounts of alcohol rather consistently, often beginning the first day. Others (Fig. 2) avoided the consumption of alcohol at first but after a

time drank more and more until after a few weeks a large part of their fluid intake was 10% alcohol. Still others (Figs. 3 and 4) avoided the consumption of substantial amounts of alcohol over a period of many weeks. A closer examination of the consumption curves shows them to be highly individual in character. In some animals the consumption was relatively steady, in others it tended to fluctuate widely. In Fig. 3 it will be noted that the consumption was consistently higher than that

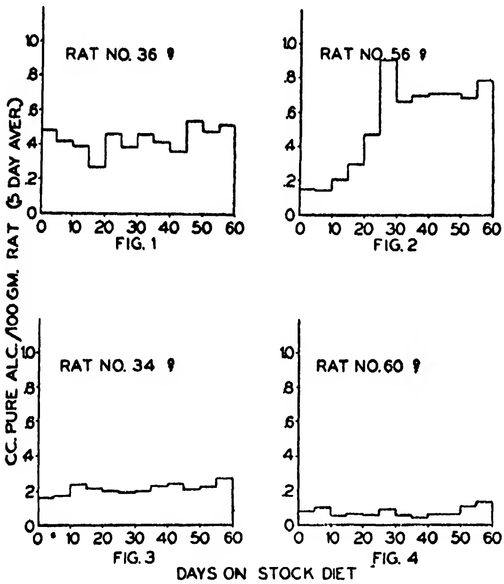


FIG. 1 FIG. 2 FIG. 3 FIG. 4

FIGS. 1–4. Representative individual responses of rats as shown by *ad lib.* selection of water or 10% ethyl alcohol.

shown in Fig. 4 but continued for a long period at a very moderate level. Further evidence as to the distinctive character of the individual responses will be presented later.

Effect of Diet in Experiments with Groups of Animals

When groups of animals were placed upon diets A and B instead of stock diets, the average responses were found to be dependent upon the type of diet used, in confirmation of the earlier work of Mardones, Segovia and Onfray (9), and Brady and Westerfeld (10). These workers have found that groups of rats on restricted diets consume larger amounts of alcohol than when the restricted diets are supplemented with B vitamins and liver and yeast extracts. Mardones, Segovia and

Onfray have postulated the existence of an unknown principle, "Factor N," which controls appetite for alcohol. Brady and Westerfeld also observed the efficacy of a liver extract preparation in decreasing alcohol consumption under certain conditions, but after the alcoholic consumption became relatively large they were unable to observe more than a temporary diminution of consumption caused by its addition to the diet.

In Tables I and II are included summaries of the data on alcohol consumption with respect to two strains of rats on diet A and diet B.

TABLE I

Alcohol Consumption of Rats and Mice

Type animal studied	Number of animals	Diet[a]	Av. alcohol consumption 25–40th day as cm.^{3}/100 g. animal/day	Range of alcohol consumption	Mean of deviation from mean	Percentage of deviation from mean
Strain "O" rats	10	A	.93	.48–1.39	.21	23
Strain "O" rats	10	B	.17	.07–0.29	.05	30
Strain "H" rats	41	A	.73	.18–1.83	.25	34
Strain "H" rats	38	B	.27	.07–0.63	.11	41
Dba Strain mice	10	A	.29[b]	.24–0.38	.036	12
C_3H Strain mice	9	A	.46[b]	.07–1.38	.42	91
White mice	14	A	.50[b]	.23–1.87	.27	54

[a] The rats were on the diet and had access to alcohol 25 days prior to the period covered in this table, and the mice were on the regime for 5 days.

[b] The period for the mice was from the 6th to the 14th day.

All animals were started on diets within one week from weaning.

TABLE II

Comparison of Two Strains with Respect to Their Maximum Alcohol Consumption

Strain	Number of rats	Diet	Average maximum alcohol consumption during period on diet	Range	Av. no. days to reach maximum	Range of days (indiv. rats)
"O"	26	A	.94±.24[a]	.58–1.54	53	35–81
"H"	39	A	1.33±.35[a]	.72–2.17	52	35–81
"O"	26	B	.35±.14[a]	.08–0.75	81	62–120
"H"	42	B	.47±.23[a]	.03–1.00	61	53–87

[a] These values are the mean deviations from the mean, and not standard deviations.

It will be noted that diet A induces far greater alcohol consumption than diet B and that the maximum consumption (Table II) is reached earlier on diet A than on diet B.

While a detailed analysis of the total water consumption records has not been made, it appears evident that (1) the total fluid consumption of the rats figured on a weight basis decreased with increase in weight, and (2) when the total fluid consumption of groups of rats of the same weight exhibiting high and low alcohol consumption are compared, there is no striking difference.

Genetic Basis for Alcohol Response Patterns

It has previously been shown that ordinary inbred strains of laboratory animals are by no means homogeneous with respect to their nutritional requirements, and that substrains of such animals with different levels of requirement for specific vitamins can be bred (11,12). From the data presented in Table I several pertinent observations may be made: (1) While "Strain O" and "Strain H" rats both consume much more alcohol on diet A than on diet B, the contrast between the two diets is significantly greater in the case of "Strain O" rats. (2) On diet A "strain O" rats consumed 27% more alcohol than did "strain H" rats. (3) On diet B ,however, the situation was reversed; the "strain H" rats consumed 60% more alcohol. (4) Variability within the group is relatively high in the case of the two strains of rats and in the C_3H mice and the white mice, whereas the variability is relatively low among the dba mice.

These observations find a ready explanation on the basis of genetic differences and no other explanation has suggested itself. In the case of the dba mice which are being used extensively in our laboratories in cancer investigations, brother-sister matings have been strictly adhered to and this appears to account for the low variability in this group. The C_3H mice ordinarily are handled in the same manner but breeding difficulties have been encountered and brother-sister matings had to be abandoned until the difficulties could be eliminated. The two strains of rats and the white mice are of the sort commonly used for nutritional investigations and no special precautions have been taken in connection with their breeding.

It should be noted that the extreme variability exhibited by the C_3H mice is due in part to the fact that, for these animals, diet A is

very deficient. The experiment involving these mice could not have been extended much longer because of inadequacy of the diet. Strain differences in nutritional requirements of this sort have doubtless a genetic origin.

The existence of wide variations in individual responses (Tables I and II), which, however, are minimized in the dba mice, can hardly be explained except on other than a genetic basis, and the high strain variability with respect to treatment to be discussed later, offers further strong evidence as to the importance of genetic factors in determining the responses to alcohol.

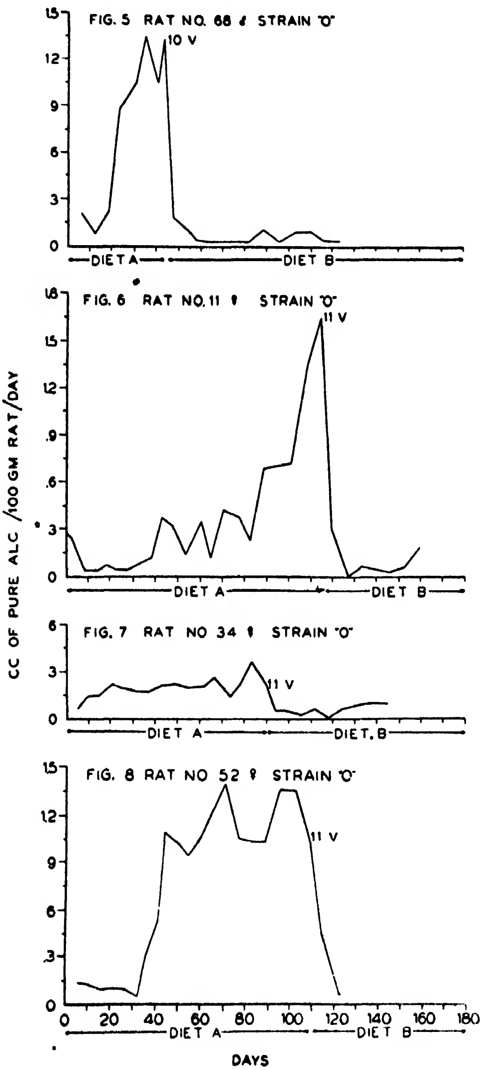
Control of Alcohol Appetite in Animals

Perhaps the most striking evidence of genetic variability was obtained in connection with the treatments which were instituted to overcome the alcohol appetite of the rats. Animals of "Strain O" and of "strain H," for example, responded in a significantly different manner when they were administered 10 B vitamin by mouth and the anti-pernicious anemia vitamin by injection. In every one (100%) of the 24 rats of "strain O," the alcohol consumption was decreased to a low level by this treatment. In 70% of these rats no difficulty was encountered in keeping the alcohol consumption at a low level. In the other 30%, there was a tendency to revert to a higher level as in the work of Brady and Westerfeld, which tendency, however, in our experiments could usually be overcome by repeated vitamin administration.

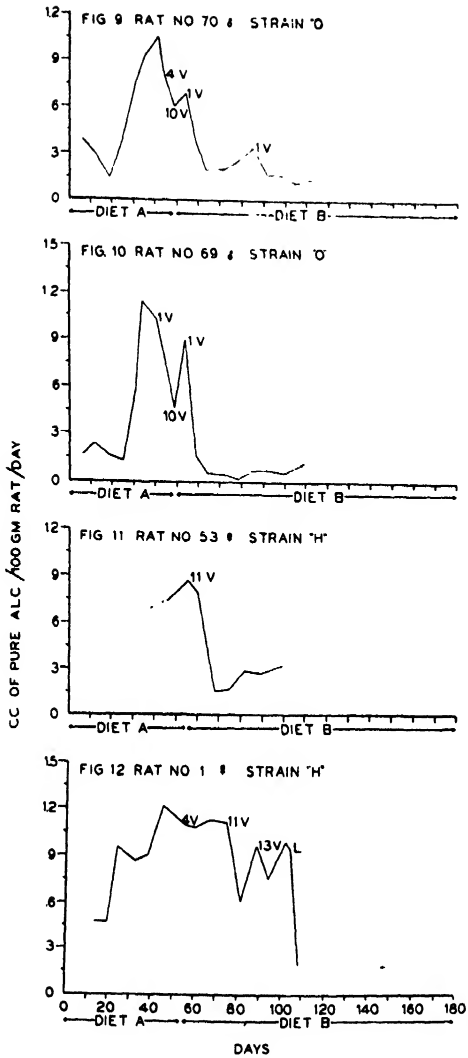
Among the 45 rats of "strain H" treated in the same manner, only 3, or about 7%, showed a diminution of alcohol consumption to a low level. In view of the relatively large number of animals used and the consistency of the results, there seems no question as to the significance of the observed differences between the two strains.

A large number of rats have been treated in the course of our investigation. Typical results can best be presented in the form of a series of individual consumption curves on which are indicated in code the treatments that were instituted. These results show that, for individual rats on the indicated diets, different nutritional factors enter into the creation of an appetite for alcohol. For example, one rat of "strain O" (Fig. 5) was dramatically and permanently cured of its desire for alcohol by the oral administration of 10 known B vitamins alone. At the other extreme is Rat No. 1, Strain H (Fig. 13), the alcohol consump-

tion of which remained at a high level, even after 3 other vitamins had been administered, *viz.*, vitamins A, E, and the anti-pernicious anemia vitamin. When some linseed oil was added to the diet, however, the alcohol consumption promptly fell to nearly zero. Various intermediate variations are depicted in Figs. 6 to 12. Fig. 6 represents the consumption curve of a rat which was very slow in developing an appetite which, however, was dramatically abolished by the administration of 10 known



FIGS. 5–8. Effect of diet upon individual alcohol consumption levels.



FIGS. 9–12. Effect of diet upon individual alcohol consumption levels.

B vitamins plus the anti-pernicious anemia vitamin. Fig. 7 has to do with a rat which drank steadily over a long period at a low level. Its appetite too was abolished by a similar vitamin treatment. Another rat (Fig. 8) remained at a high level of intake for nearly 7 weeks, when the administration of 11 vitamins promptly brought the consumption down to nearly zero. Rat No. 70 (Fig. 9) is one for which the anti-pernicious anemia vitamin appears to be unusually important. This is

somewhat true of Rat No. 69 (Fig. 10) which showed an initial response to the anti-pernicious anemia vitamin alone, showed an increase when the B vitamins were first administered, but subsequently a drop to approximately zero when a second treatment with the anti-pernicious anemia vitamin was given. Fig. 11 depicts the case of a rat of "strain H" which had its appetite greatly decreased by the administration of 11 vitamins. This rat had access to alcohol for 55 days previous to the treatment, but its consumption record for the earlier period was not taken.

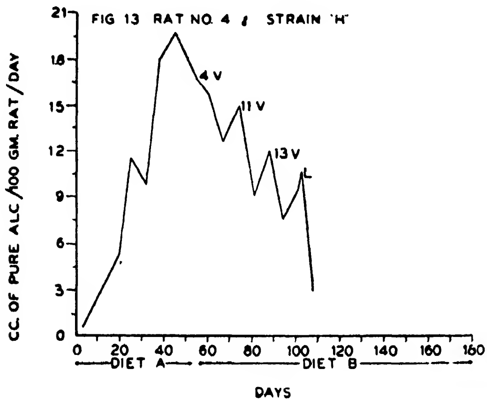


FIG. 13. Effect of diet upon individual alcohol consumption levels.

In Fig. 12 and 13 it will be noted that additional vitamin A administration appeared to be effective in bringing about a material diminution of alcohol consumption in "strain H" rats. A diminution was observed in 2 days in every one, 100%, of the 31 rats furnished additional vitamin A along with the other 12 vitamin supplements already furnished. In another series of experiments the addition of linseed oil to the diet caused a marked diminution of the alcohol consumption in 4 out of 6 rats. Two such cases are depicted in Figs. 12 and 13.

Our experimentation in the control of alcohol appetite in mice has not been extensive. The experiments which have been performed indicate that the problem with mice is not essentially different than with rats. Only 6 mice have been treated, and these for only 4 days at the date of this writing; of these, 5 diminished their intake, one remained unchanged. The average diminution for the 6 was 54%. One had diminished its consumption to zero.

It is clear from our experience that extended studies must be made before it will be possible to abolish with complete success the alcohol appetite of very individual laboratory animal regardless of its peculiar inheritance. We have not yet investigated in the general field of amino acids or minerals; it may be that, in some animals, a partial genetic block causes a high requirement for one of these and that such deficiency induces in some unknown manner an abnormal appetite for alcohol. Our experience convinces us, however, that it will be possible by an extension of the means we have already employed to abolish the appetite for alcohol in 100% of laboratory animals.

Habituation Experiments

It has been tacitly assumed, in connection with a number of studies (13), that animals can be habituated to alcohol simply by forcing them to drink it over a period of time. According to the genetotrophic concept this should not be the case for all the animals if the diet of the animals is a reasonably satisfactory one.

While our experiments on this subject have not been carried very far, certainly they indicate strongly that habituation, particularly in the sense of addiction, is not brought about by the simple procedure that has had wide use.

TABLE III

Effect of Forced Alcohol Consumption ("Habituation") on Voluntary Intake of Alcohol

Group	No. of rats	Diet	cc. Alcohol/100 g. rat/day	Range	Mean of deviation from mean	Percentage of deviation from mean
"Habituated"	9	B	.09	.06–.13	±.01	11
"Habituated"	8	Stock	.06	.05–.09	±.01	17
Control	18	B	.15	.07–.25	±.05	33
Control	8	Stock	.32	.07–.78	±.18	51

A group of 9 weanling rats was placed for 29 days on diet B and furnished as the only available fluid 10% alcohol. A similar group of rats was placed on the same regimen, except that a stock diet was used instead of diet B. Two control groups (one on each diet) were placed upon the same regimen except that they were given a choice from the start between 10% alcohol and water.

At the end of the 29-day period the 4 groups all had a choice between water and 10% alcohol as in the earlier experiments described. The results presented in Table III show that animals which are forced to take 10% alcohol for a period of time are by no means led to take large amounts by choice. There is the strong suggestion that, when rats on essentially adequate diets are forced to drink alcohol, they actually develop a dislike for it and drink less than if they had been given a choice from the beginning. In any event, this procedure, which has often been used in animal studies on alcoholism, is clearly not promising as a means of producing compulsive drinkers.

Theory of Alcoholism

The theory suggested and supported by these experimental findings may be outlined as follows: Appetite for alcohol is a physiological perversion based upon incompletely satisfied nutritional needs. We know relatively little as to the mechanism whereby physiological changes in the body induce special appetites, but that special appetites are induced by the internal environment is undeniable. Alcoholic craving, according to our theory, develops as an overpowering drive in certain individuals as a result of their unusually high requirement for one or more specific nutritional entities, such as the B vitamins, and because this high need is not fully satisfied by the common foods which are consumed. The use of large quantities of refined foods would, according to this theory, contribute to alcoholism; and the consumption of alcohol itself, in addition to developing a taste, would promote the appetite and start a vicious cycle because it crowds out of the diet wholesome foods which normally contribute to the various needs.

There may or may not exist a few specific lacks (known and unknown) which are generally associated with the compulsive drinker's appetite. Our experiments with rats indicate that widely different deficiencies may contribute directly or indirectly in producing an appetite for alcohol. Among the controlling factors are various of the known B vitamins, the anti-pernicious anemia vitamin (vitamin B_{12}), vitamin A, and unsaturated fat acids (linseed oil). Our knowledge of precisely how the anti-pernicious anemia vitamin is obtained and assimilated from natural diets is limited. It was administered to the animals by intraperitoneal injection in order to insure its absorption. The failure to assimilate this or some other vitamin might lie at the basis of certain cases of alcoholism.

Our experimental studies strongly suggest that the *abundant* satisfaction of *every* nutritional need will abolish alcoholic appetite. It should be emphasized that in providing for every nutritional need the chain is as strong as its weakest link, and satisfying every need except one should be expected to be completely ineffective. Furthermore, due partly to differences in assimilation, bacterial action in the intestines, *etc.*, as well as distinctive features in the pattern of intermediate metabolism, the requirement for a given vitamin or other nutritional entity by a particular individual may actually be far above what is considered normal. This will make it more difficult than might be supposed to insure to him an adequate supply of every potential nutritional factor.

It is true that the treatment of alcoholism has often included the administration of supposedly generous amounts of B vitamins. A sufficient cause for the failure of vitamin therapy as a treatment for alcoholism in the past is the omission of the anti-perniciuos anemia vitamin as well as sufficient amounts of certain other vitamins which in individual cases may be necessary links in the chain. Commercial vitamin preparations are often inadequate and poorly balanced (14), and the idea back of whatever therapy that has been used has been to remedy deficiencies arising because of excessive alcoholic consumption. No one, so far as we known, has ever seriously considered a nutritional deficiency to be a cause of alcoholism, and no attempts have been made, therefore, to correct an underlying deficiency. Requirements in such cases may be very high or hard to satisfy, and haphazard administration of "vitamin pills" and self-medication by uninformed laymen can be expected to be completely ineffective, or even harmful or dangerous.

The effects of very high alcoholic consumption have not as yet been studied in our investigation. On a body-weight basis, the maximum consumption observed under our conditions is equivalent to 3.7 qt. of 90 proof liquor per day for an average man. In proportion to the total food consumption of a rat, however, the amounts are much smaller. It may be that continued nutritional deprivation associated with high alcoholic consumption does irreparable damage to the individual and that, for this reason, advanced stages of alcoholism will be incurable by satisfying all the nutritional needs. We may hope that is not so, and the presumptive evidence is against this idea.

The theory which we have set forth, if valid, emphasizes the importance of good nutrition and the desirability of fulfilling the nutritional needs of everyone—not only those who have average requirements. In calculating nutritional needs, a far larger factor of safety than is generally used may well be in order. It seems, on the basis of our experiments, very likely indeed that consistent good nutrition from childhood on will strongly militate against alcoholism. Regardless of the efficacy of any treatment which has its basis in the theory propounded, the prophylactic effects of good nutrition are strongly indicated. Good nutrition may, however, mean in some cases nutrition designed for the needs of a particular individual with specific requirements that are unusually high.

An interesting manner in which the proposed theory fits the facts has to do with the relationships between age and alcoholism. If an indivi-

dual becomes an alcoholic at an early age, before 28 (15), up to now there has been practically no hope for him. In general, those who become alcoholic later in life are much more susceptible to treatment. According to our theory, those individuals who become alcoholics early are those who have the highest requirement for some specific nutritional factor; the resulting deficiency obviously shows up earlier and the possibility of its being met or even half-way met is remote. Those who become alcoholic in later years do not have such marked deficiencies and the urge can perhaps be broken by psychological means.

Study of alcoholism from the point of view which we have developed is in its infancy, and much further research will be required to clarify the problem and to test various ramifications of the theory. One line of research must center on the problem of precisely what nutritional deficiencies may be responsible for inducing an appetite for alcohol.

Another research field of large proportions has to do with developing means of assessing individual metabolic patterns, so that something other than a "shotgun" therapy can be used. We believe that it will eventually become possible through the efforts of many investigators in many laboratories to determine, in the case of any specific alcoholic, the biochemical roots of his particular difficulty, and, on this basis, to formulate an adequate treatment. For adequacy in this regard, it is essential that *every nitritional need be met simultaneously.*

It should be noted also that, even if one has relieved the appetite of an alcoholic, no lasting benefit would result were he allowed to go his way, become deficient again and relapse into the condition of alcohol craving again. How much danger will exist in this direction, only experience can show. It is obvious, however, that a one-time alcoholic should continue to have his nutritional needs satisfied continuously throughout life.

Finally, another vast field of research which is opened up by the theory we have proposed is the possible relation of genetotrophic factors to other diseases of obscure etiology, which have previously been mentioned.

Acknowledgments

We gratefully acknowledge our indebtedness to our colleagues and assistants, to Helen Kirby, who has carried forward the investigation of individual metabolic traits, and to Janet Reed, Nellie Keffer, William Brown and Gene Rich, whose technical assistance has been invaluable.

Summary

Evidence is presented, based upon animal experimentation, that alcoholism is a genetotrophic disease, *viz.*, a disease in which genetic factors and nutritional deficiency together are etiological agents. It has been found possible, by the administration of nutritional factors, to control the appetite for alcohol in laboratory animals. It is suggested that genetotrophic factors may be important in may diseases of obscure etiology.

Code for Alcohol Consumption Curves

1V

B_{12}—5 units of 20 unit anti-pernicious anemia liver preparation given intraperitoneally each day for 5 consecutive days.

4V

	mg./day for 5 days
Thiamine	0.3
Riboflavin	0.5
Calcium pantothenate	3.0
Folic acid (Folvite)	0.1

5V

Same as 1V + 4V, except that the B_{12} was partially purified.

10V

Same as 4V with the addition of:

	mg./day for 5 days
Pyridoxine	0.15
Choline	100.0
Inositol	3.0
p-Aminobenzoic acid	3.0
Nicotinic acid	1.0
Biotin	0.01

11V

Same as 1V + 10V.

13V

Same as 11V with the addition of:

	units/day for 5 days
Vitamin A	500
Vitamin D	100

L

Same as 13V with the addition of:

Linseed oil. . . . . 5% by weight to total diet.

Note: In the alcohol consumption curves the experimentally determined values are plotted. These are uncorrected for spillage and evaporation. Hence, a low value may actually represent zero consumption.

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The Reaction of Tobacco Mosaic Virus with Formaldehyde. I. Electrophoretic Studies [1]

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Introduction

In 1938, Ross and Stanley (1) reported that reaction between 2% tobacco mosaic virus (TMV) and 2% formaldehyde, buffered with $M/10$ phosphate to pH 7.0, causes the infectiousness of the virus to be destroyed and causes a decrease in the amount of color developed with ninhydrin and with Folin's phenol reagent at pH 7.7. Furthermore, these workers found that, if virus which had been inactivated was subsequently dialyzed for 3 days against a dilute pH 3.0 buffer, the lesion-count on *N. glutinosa* was increased. The increased lesion-count was accompanied by a reversal of the chemical changes, for the amount of color developed by both ninhydrin and the phenol reagent was found to be increased by the dialysis. Kassanis and Kleczkowski (2) failed to observe the reversibility of the reaction and were unable to correlate the chemical changes effected by formaldehyde treatment with the loss of infectiousness. More recently, Fraenkel-Conrat *et al.* (3) reported that formaldehyde treatment at pH 7.0 decreases the availability of the tryptophan and tyrosine residues to the Folin reagent, but that formaldehyde does not actually combine with these groups. Therefore, it seemed worthwhile to investigate the electrophoretic changes resulting from the treatment of TMV with formaldehyde, and to repeat those experiments of Ross and Stanley which indicate that the infectivity of

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[2] Some sections were abstracted from a thesis submitted by Marie A. Fischer to the Department of Chemistry in partial fulfillment of the requirements for the Ph.D. degree.

[3] Contribution no. 721 of the Department of Chemistry and 5-p-49 of the Department of Physics, University of Pittsburgh.

formaldehyde-inactivated virus can be increased by dilute pH 3.0 dialysis.

Materials and Methods

The juice expressed from infected Turkish tobacco plants was passed through a Sharples Supercentrifuge. Ninety per cent of the infectious material was collected on the cylinder and, after suspension in $M/10$ phosphate, pH 7.0, the material was subjected to a low and high speed centrifugation cycle (4). The cycle was repeated 4 times. The final supernatant fluid was colorless and, on the basis of activity measurements, a 100-fold concentration of virus was effected. The protein content of the purified solution was determined by Kjeldahl analyses for nitrogen, and infectivity was measured by the half-leaf method of inoculation (5), using *N. glutinosa* as the test plant. So that approximately equal numbers of local-lesion would be caused by treated and control samples, several dilutions of each sample were usually made and compared by the half-leaf method and a Latin-square pattern for inoculation.

Formaldehyde-treated TMV was prepared by allowing solutions containing 2% virus and 2% formaldehyde to react at room temperature (1). In the pH 7.0 experiments the reaction was carried out in $M/10$ phosphate, and in the pH 4.0 experiments in 0.077 M Na_2PO_4-0.063 M citric acid. To stop the reaction, the formaldehyde was removed by dialysis for 6 hours against cold, distilled water in a rocking dialysis apparatus.

For the prolonged pH 3.0 dialyses, the procedure of Ross and Stanley (1) was followed as closely as possible. The formolized virus was dialyzed against flowing 0.001 M phosphate-citrate-HCl buffer at pH 3.0 in a rocking dialysis apparatus. The temperature was kept between 5 and 10°C. The samples were subsequently dialyzed to pH 7.0 with $M/10$ phosphate, analyzed for nitrogen, and inoculated at suitable dilutions against a control sample of virus. In all cases the inactive virus and the aliquot dialyzed at pH 3 were inoculated at the same protein concentrations.

The electrophoretic analyses were carried out in the apparatus described by Tiselius (6) as modified by Longsworth (7). All of the preparations were diluted to a protein concentration of 0.5% and were dialyzed for 3 days against two 1 l. and a third 2 l. portions of pH 7.00, ionic strength 0.2, KH_2PO_4-K_2HPO_4 buffer. The samples migrated in an electric field of 4.5 volts/cm., with continouus compensation, usually for 24 hr. Mobilities were determined for several preparations by measuring migration distances after 2 hr. of electrophoresis without compensation under otherwise similar experimental conditions. In each case the current was reversed, the boundaries were returned to their initial positions, and the mobilities were calculated from the averages of the migration distances of both boundaries.

Experimental Results and Discussion

The Reaction at pH 7.0

It has been shown (1,8) that formaldehyde-inactivation of tobacco mosaic virus, in a neutral buffer, and at room temperature, follows the course of a first-order reaction. Table I shows that, under the same

TABLE I

The Effect of Formaldehyde Treatment on Tobacco Mosaic Virus[a]

Time of HCHO treatemnt[a] (*hr.*)	Activity remaining *per cent*	Anodic mobility *cm.²/volt-sec* $\times 10^5$
0	100	7.20
6	12.1	7.31
6	10.8	7.37
12	1.63	7.43
24	0.25	7.48
24	0.078	7.58

[a] Each analysis is for a separate experiment carried out at room temperature for the time shown.

conditions of treatment, an increase in the anodic mobility of the virus occurs and the increase in mobility is dependent upon the time of contact between formaldehyde and the virus. Since a first-order loss of activity is frequently interpreted as implying an "all-or-none" mechanism, it seemed desirable to determine whether the electrophoretic mobility of all the virus particles had been changed, or whether some had undergone a change in mobility and others had not. To study this problem, samples which had been inactivated to varying degrees were subjected to electrophoresis at pH 7.0 for approximately 24 hr. Fig. 1 shows typical electrophoretic patterns for (A) untreated, (B) 72% inactivated, and (C) 99% inactivated virus after prolonged electrophoresis. All of the samples inactivated at pH 7.0 showed essentially the

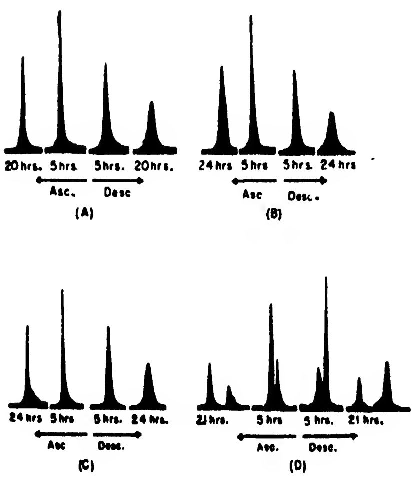

FIG. 1. Electrophoretic patterns of (A) untreated, (B) 72% inactivated, (C) 99% inactivated, and (D) a mixture of two parts 99% inactive and one part untreated virus. The virus was inactivated at pH 7.0 and the electrophoresis carried out at pH 7.0.

same degree of homogeneity as did normal virus. The electrophoretic homogeneity of the preparations was verified by studying known mixtures of active and inactivated virus. In one representative experiment shown in Fig. 1 (D), a mixture of two parts inactive and one part normal virus was easily separated into its two components. The larger fraction had the faster rate of migration, an observation consistent with the known higher mobility of formaldehyde-treated virus. It is, therefore, apparent that an approximately equal number of reactive groups on each virus particle reacts with formaldehyde at pH 7.0, and thus the reaction products retain essentially the original electrophoretic homogeneity, even though they are inhomogeneous with respect to infectivity. As is reported elsewhere (8), a theory devised to account for the kinetics of the reactions between formaldehyde and TMV leads to deductions consistent with this observation.

The Reaction of pH 4.0

Formaldehyde inactivation at pH 4.0 seems to be a first order reaction with a reaction velocity constant of 0.45 reciprocal hours at room temperature. In this respect, the reaction at pH 4.0 is similar to that at pH 7.0 (1,8). However, the chemical changes appear to be different. At pH 4.0, insoluble material appears during the course of the reaction and, after about 12 hr., gel formation is evident. These changes were not observed in a control sample of virus held at pH 4 or in virus inactivated at pH 7.0. It has already been shown that formaldehyde

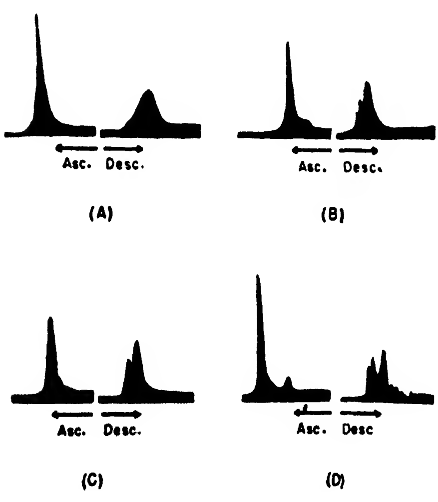

FIG. 2. Electrophoretic patterns of TMV treated with formaldehyde at pH 4.0. Electrophoresis was carried out at pH 7.0 for 24 hr. (A) represents control TMV, (B) after 2 hr. HCHO treatment, (C) after 6 hr. HCHO treatment, and (D) after 24 hr. HCHO treatment.

inactivation at pH 7.0 results in a single component by the criterion of electrophoresis at pH 7.0. Therefore, for comparison, virus was treated with formaldehyde at pH 4.0 and subsequently electrophoretically analyzed at pH 7.0. Before electrophoresis, each sample was centrifuged at 3500 r.p.m. for 15 min. to remove any large particles of insoluble material. Fig. 2 shows the electrophoresis patterns obtained for several samples of virus treated in this manner. The control sample, which was subjected to the same pH change but was not treated with formaldehyde, appeared to be essentially homogeneous. The samples

TABLE II

The Effect of Prolonged pH 3.0 Dialysis on the Activity of Formaldehyde-Inactivated TMV

(1) Conc. of sample	(2) Activity remaining before pH 3 dialysis	(3) Activity remaining after pH 3 dialysis	(4) Ratio of (3)/(2)
g./cc.	*per cent*	*per cent*	
10^{-4}	9.0	17.0	1.9
10^{-4}	7.6	7.9	1.0
10^{-4}	6.5	9.0	1.4
10^{-4}	17.0	16.0	0.9
10^{-4}	13	13	1.0
10^{-4}	13	16	1.2
5×10^{-3}	0.078	0.25	3.3
10^{-3}	0.93	1.6	1.7
10^{-3}	1.23	1.26	1.0
10^{-3}	1.05	1.19	1.1
10^{-3}	0.006	0.017	3.1
10^{-2}	0.019	0.084	4.4
10^{-2}	0.069	0.129	1.9

treated with formaldehyde at pH 4.0 at room temperature gave inhomogeneous patterns which were anomolous because the inhomogeneities were not symmetrical with respect to the ascending and descending boundaries.

The Effect of Prolonged pH 3.0 Dialysis on Formaldehyde Inactivated TMV

The data of Table II support the observation of Ross and Stanley (1) that prolonged pH 3.0 dialysis of formaldehyde-treated virus causes an increase in the activity of the virus preparation. It is unlikely that errors in the testing method can explain the difference in lesion-count

between inactivated virus and the aliquot which was subsequently dialyzed at pH 3.0. Errors in the method should give rise to random variation in the relative activities of the samples. In that case, the mean of the ratios of the relative activities of the sample before and after pH 3 dialysis should be one. Actually, the mean for all of the experiments was 1.84, and the standard deviation of the mean was $\pm$ 0.31. Since the deviation from one is 2.7 times the standard deviation of the mean, it is improbable that the increased lesion-count following the pH 3 dialysis is the result of random error.

It was not possible to detect electrophoretic mobility differences between inactive virus and the same sample subsequently dialyzed for 3 days at pH 3.0. Furthermore, mixtures of the two did not seqarate into fractions during 24 hr. of electrophoresis at pH 7.0. If the prolonged pH 3.0 dialysis affects groups responsible for the net charge on the particle, the mobility change is so small that it is undetectible under the conditions of the experiment.

Summary

1. Reaction between tobacco mosaic virus and formaldehyde at pH 7.0 resulted in an electrophoretically homogeneous preparation, regardless of the time of formaldehyde treatment.

2. When formaldehyde treatment was carried out at pH 4.0, the preparations appeared inhomogeneous when the electrophoresis was carried out at pH 7.0.

3. Dialysis of inactivated virus at pH 3.0 for 3 days caused an increase in the lesion-count when the infectiousness was tested by inoculation on *N. glutinosa*.

4. Under the conditions of these experiments, it was not possible to detect a change in the electrophoretic mobility of inactive virus after it had been dialyzed at pH 3.0 for 3 days.

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An Interpretation of the Contradictory Results in Measurements of the Photosynthetic Quantum Yields and Related Phenomena

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I. Contradictions in the Values Measured for the Photosynthetic Quantum Yield

Warburg and Negelein (1) were the first to measure the quantum yield of photosynthesis. They found that 4 light quanta are sufficient to reduce one CO_2 molecule. Thermodynamic considerations made it difficult to understand this high yield, but their measurements remained uncontested for many years. Doubts arose concerning them when new observations, made in Farrington Daniels' laboratory in Madison (2), consistently gave much lower quantum yields with an upper limit of about 1/10 instead of 1/4. By copying the original observation method exactly, Rieke (3) was able to get the same quantum yield (1/4) as Warburg and Negelein. However, when the algae (*Chlorella*) which served as a plant material for all of these measurements were kept in an alkaline buffer solution, the yield had an upper limit of $\sim 1/8$. An explanation of these contradictory results was presented by Emerson and Lewis (4), who found that, in their own careful measurements, made under conditions supposed to be identical with Warburg and Negelein's ,an outburst of CO_2 at the beginning of illumination and a corresponding uptake of that gas during the following dark period changed the photosynthetic quotient to such an extent that apparent quantum yields even larger than 1/4 could be calculated. However, the rate of photosynthetic oxygen production gave a yield of $\sim 1/10$ whether measured by use of a CO_2 buffer or by Warburg's well known two-vessel method. This explanation of the differences was generally accepted in this country, especially as a number of observers using a diversity of methods all found $\sim 1/10$ as the upper limit of the

quantum yield (5). Recently, Warburg (6) has rejected Emerson's criticism. His new measurements give the same quantum yields as his old ones and a photosynthetic quotient near unity. However, the quotient and the quantum yield were measured under different conditions, and it is thus still an open question to what extent the CO_2 outburst (Emerson effect) may have falsified Warburg's measurements. Because Warburg and Emerson used comparable experimental methods, emphasis will be placed on the contradictions between their results.

The present writer, who was priviliged to have oral discussions with Warburg and with Emerson, finds it hard to accept the point of view that only Warburg's method under special conditions will permit the algae to reduce CO_2 with a quantum yield of 1/4 when all other observations systematically give $\sim$ 1/10 as the highest value. On the other hand, it is not certain that the occurrence of the Emerson effect is the main cause of the difference between Enerson's and Warburg's new results, because this effect, while undoubtedly present in both observations, seems to be smaller in Warburg's new experiments than in Emerson's. The main part of the CO_2 outburst during irradiation occurs during the first minutes of illumination, while the corresponding uptake of CO_2 during dark periods shows a slower decay. If we compare only those measurements of Warburg with Emerson's in which the precaution is taken of not using these first minutes of pressure changes in each light and dark period for the calculations, we still find differences of about 100%; *i.e.*, apparent quantum yields between 1/4 and 1/6 according to Warburg and 1/10 according to Emerson and Lewis.

These facts raise doubts as to whether the Emerson effect suffices for the explanation of Warburg's results. Experiments are now in progress in Warburg's and Emerson's groups in which the main emphasis is laid upon the question of whether, under the special conditions of Warburg's experiments, a gas exchange with a photosynthetic quotient of $\sim$ 1 is possible giving the result of a quantum yield of $\sim$ 1/4.[1] However, not only this problem is at stake, because there are further differences, discussed later, between the results of Warburg's measurements and those of Emerson and Lewis (and the other observers who measured the quantum yield with different methods). They seem to indicate that Warburg's results differ not only quantita-

[1] Compare the note added in proof at the end of the paper.

tively but also qualitatively from those of the others. A similar situation exists for another basic problem of photosynthesis—the identification of the chemical nature of photosynthetic intermediates. Two groups engaged in studying these compounds with the help of the radioactive isotope, C^{14}, have obtained entirely contradictory results.

II. Proposal to Interpret the Differences as Caused by Variations in the Permeability of Chloroplast Membranes to Respiration Intermediates

The present writer is inclined to seek a way out of these dilemmas by the following assumption: Normally the process of respiration in the cells has another chemical pathway than, and is locally separated from, the process of photosynthesis in the chloroplasts. However, under special conditions, the chloroplast membranes become permeable to intermediates of respiration. In that case, the processes of dark and light metabolism become intertwined and interfere with each other. Correspondingly great changes of both processes occur during illumination. They may be of brief duration if the photosynthesis rate is much higher than that of repiration, or permanent when the photosynthetis gas exchange is smaller, or not much higher, than that of respiration. The following chapters contain an analysis of the inconsistencies in the measurements of the quantum yield based on this idea.

We introduce the assumption that Warburg's high quantum yield may be connected with the reduction of respiratory intermediates rather than with the reduction of CO_2. That is possible because Warburg's measurements are carried out under conditions where the photosynthetic rates are smaller than or, at best, comparable to, the respiration rates. That such a process may occur and influence the measurements of the quantum yield of photosynthesis is by no means a new idea. It was proposed a long time ago by different authors but such statements did not receive much attention, nor were attempts made to give a detailed explanation of why the effect occurs only under special conditions, or how it is connected with other observations in the field of photosynthesis. It may, therefore, be useful to examine this possibility more closely. We will see that the assumption is not only able to reconcile Warburg's quantum measurements with those of others, but also agrees with results of an entirely different nature.

III. Evidence Supporting the Hypothesis that the Gas Exchange Measured by Warburg is Due to Photochemical Reduction of Respiration Intermediates

1. Quantum Yield and Photosynthetic Quotient of This Process

The first questions which arise are the following: Will reduction of intermediate respiration products proceed with a quantum yield of $\sim 1/4$? Will the gas exchange connected with this process have the same quotient as the photosynthesis of CO_2? To answer these questions, we do not need to enter into a detailed discussion concerning the exact chemical nature of the respiration products, because we know that in the overall process of respiratory oxidation of carbohydrates one oxygen molecule is consumed for each CO_2 molecule formed. We, therefore, may speak only of carbohydrates, of half-oxidized respiration products and of CO_2. The term carbohydrates includes all substances of the general formula $(CH_2O)_n$. Half-oxidized products are substances containing a group which can be restored to the oxidation state of a carbohydrate by the addition of two hydrogen atoms, and CO_2 stands for free CO_2 or for substances in which a carboxylated group may be reduced photochemically. These latter groups need 4 hydrogen atoms for reduction to the carbohydrate state. Obviously, half as many quanta per molecule as are used for the reduction of CO_2 should then suffice to reduce the half-oxidized products and one-half molecule of oxygen will be evolved for each of these molecules reduced to carbohydrate. The photochemical removal of half-oxidized molecules from the course of respiration means that oxidation by respiration proceeds only half way. Thus, every half-oxidized molecule reduced photochemically will prevent the consumption of one-half molecule of oxygen by respiration. The evolution of CO_2 will be diminished by a whole molecule. In other words, the process would add one oxygen molecule and take away one CO_2 molecule from the balance sheet of respiration for every 4 quanta absorbed by the photosynthetic apparatus. If, far below the compensation point, the photochemical gas exchange should consist solely in the reversal of respiration, a quantum yield of $\sim 1/4$ with a normal photosynthetic quotient would result.[2]

[2] Correspondingly an apparent quantum yield of $\frac{1}{2}$ should be observed if respiration products reducible by one hydrogen atom are photochemically diverted from the course of respiration.

With rising light intensity, the contribution of the reduction of respiratory intermediates to the overall photosynthetic gas exchange should become smaller, while the contribution made by photosynthesis of CO_2 will rise. Correspondingly, the quantum yield should gradually fall with rising light intensity to a limiting value equal to the quantum yield of CO_2 assimilation, a value half as great as for the reduction of the respiratory intermediates. The shape of the curve, quantum yield *vs.* light intensity, cannot be predicted without further hypothesis. Let us introduce the assumption that, in Warburg's algae, the membranes separating the chloroplasts from the rest of the cell are so easily permeable to these respiratory intermediates that at higher light intensities they all diffuse into the photosynthetic apparatus instead of being further oxidized by respiration. The occurrence of a quantum yield of 1/4 at very low light intensities may be taken as an indication that reduction of intermediates occurs preferentially to that of CO_2. Thus, we may estimate that the light intensity necessary to divert all respiratory intermediates from the course of respiration would probably lie about 3 times higher than the one necessary to reach the compensation point. The apparent quantum yield measured with light of that intensity would then be $\sim 1/7$. While the extreme case of total diversion of intermediates from the course of respiration may not be entirely realized in Warburg's measurements, it seems at least to give an approximate description of the situation.

Warburg uses a great surplus of algae in order to guarantee that at all times, in spite of the shaking of the vessel, all of the incident light is absorbed. As a result, only a small fraction of the algae is exposed at each moment to the full intensity of the incident light; in fact, the bulk of them receive no light. The stirring quickly exchanges the layer of exposed algae with others emerging out of the darkness. In effect, this situation constitutes a kind of flash illumination of the algae in which the duration of the flash is several times shorter than that of the dark period. Even though, during the brief illumination period, the respiratory intermediates may be more quickly removed by photochemical reduction than they can be replaced by diffusion into the chloroplasts, this can be compensated for by diffusion occurring during the dark periods. In Warburg's experiments we may, therefore, conclude that a relatively high percentage of all respiratory intermediates will be caught by the light and, consequently, the contribution of this reduction process should be a considerable percentage of the total photo-

synthetic gas exchange, even in the region of light intensities surpassing somewhat the ones necessary to reach the compensation point.

The following table gives quantum yields measured by Warburg as a function of light intensity. They are calculated from the steady rates of gas exchange in the light and in the dark, discarding the first 5 min. of each period (6).

Intensities of incident light Micromole quanta/min.	Quantum yields
0.158 (below compensation)	1/3.96
0.33 (below compensation)	1/4.5
0.75 (below compensation)	1/5.03
1.42 (above compensation)	1/5.56

The highest intensity used causes a gas exchange only 1.4 times that of respiration. The yields show an unmistakable tendency to become smaller with growing light intensity. Warburg explains the lowering of the quantum yield with increasing light intensity by the assumption that the rate of photosynthesis in the part of the algae exposed to the full intensity of the incident light may start to approach light saturation even before the total respiration of the bulk of the algae is compensated. Rabinowitch has pointed out that such an assumption implies light saturation values much lower than usual. Moreover, it is in contradiction to Emerson and Lewis' results who, using *Chlorella* in equal concentration, observed a linear relation between light intensity and photosynthetic rate up to values even higher than Warburg's maximum intensity. These measurements were made in an alkaline buffer solution which Warburg regards as a sufficient reason for the low quantum yield of $\sim 1/10$. However, these authors found no change in the quantum yield of the oxygen evolution if the alkaline buffer was replaced by an acid medium. Furthermore, if Warburg's arguments that alkalinity is always harmful to photosyntheiss is correct, we would expect that it would lower not only quantum yields in the region of intensities where light is the limiting factor, but also the saturation intensities and saturation values. Therefore, the rate curve should start to bend over to saturation at lower intensities in the case of Emerson's observations than in Warburg's. This also is contrary to the experimental evidence.

2. *Changes of Permeability of Chloroplast Membranes by Variation of External and Internal Conditions*

An explanation without contradictions becomes possible if we accept the hypothesis that the high quantum yields are connected

with the reduction of respiratory intermediates, and that the difference between the results of Warburg and Emerson lies in a difference in the permeability of the chloroplast membranes to the respiratory intermediates. On this basis, the lowering of the quantum yield with increasing light intensity measured by Warburg indicates that the percentage contribution of the reduction of respiratory products to the overall gas exchange starts to fall far below the compensation point. The quantum yield of 1/5.56, measured at the highest intensity, indicates that at that point it is still reponsible for $\sim 60\%$ of the photosynthetic gas exchange. In Warburg's algae the chloroplast membranes must have been very permeable; in Emerson's the membranes seem to be impermeable. It is a well known fact that great differences in permeability of membranes can be caused by somewhat different treatment of the algae (7). For instance, permeability to alkali rises with the age of the algae and the cell density, and is made especially great by prolonged anaerobicity (12). If the membranes become permeable, they do so for a variety of substances. Thus, an increase in the membrane permeability of the chloroplasts to alkali may occur under the same conditions as an increase in the premeability to respiratory products.

3. Support of the Main Hypothesis Taken from Measurements of Photosynthetic Quantum Yield Made by Other Observers

Certain observations connected with the measurements of quantum yields by Rieke (5) and Kok (5) are evidence of this correlation. Both authors observe, in acid solutions, curvature in the plot of photosynthetic rate against light intensity below and in the neighborhood of the compensation point, indicating a higher quantum yield at these intensities, and both find that the quantum yield is somewhat smaller when an alkaline buffer is used. Both authors used Warburg's manometer method for their quantum yield determinations. However, the method differs from the one used by Warburg, Emerson, and by Rieke himself in his earlier paper, in that the measurements are made with only 20–40% absorption rather than total light absorption (this method requires, in addition to the measurement of the intensity of the incident light, another one of the amount of light which passes through the algal suspension without being absorbed. Because of the light scattering, the last mentioned measurement is made with the help of an integrating sphere). Therefore, during the illumination period, all the algae are

continuously irradiated and, in a first approximation, by light of a constant intensity. Thus, even if the chloroplast membranes are permeable, a high contribution of the reduction of respiratory intermediates to the overall photosynthetic gas exchange cannot be expected in the region above the compensation point.

Most of Rieke's quantum measurements were made in alkaline buffer. Quantum yields between 1/11 and 1/12 with no dependence upon light intensity were observed under these conditions. However, in connection with rate measurements of anaerobic photoreduction in *Scenedesmus* (where hydrogen uptake replaces the oxygen evolution of photosynthesis (8)), he compared photosynthetic quantum yields and rates in slightly acid solution with those in alkaline buffer. The use of the acid medium gives a slightly bent rate curve and a quantum yield $\sim$ 20-25% higher than that given in the alkaline buffer. A few measurements of this kind made with *Chlorella* showed a similar decrease of the quantum yield due to alkalinity. The lowering of the quantum yield by alkali in these algae is, without doubt, the result of cellular damage and is not caused by removal of the respiratory intermediates from participation in photosynthesis. The latter influence could only have been responsible for $\sim$ 5% of the decrease, judging by the small deviation of the rate curve from linearity. It is, therefore, justifiable to correct, as Rieke does, the quantum yield measured in alkaline buffer to values between 1/9 and 1/10.

Kok's paper on quantum yields deserves special mention, because he not only observes the alkali damage and a rate curve in acid medium with a bend at low light intensities, but he also indicates clearly that the bend may be responsible for Warburg's high quantum yield of 1/4.[1] His plant material was *Chlorella* suspended in culture solution. Since he uses quite low algal concentrations, the pressure changes are very small at low light intensities. He, therefore, refrains from measuring the shape of the curve in this region. However, on the basis of extrapolation of the curve measured at higher light intensities, he concludes that this part of the curve must be bent. The directly observed portion of the

[1] However, it may be emphasized that we cannot agree at all with Kok's interpretation of the steeper slope of the rate curve at low light intensities. He introduces the assumption that normal respiration is stopped by irradiation and replaced by a photochemical process giving two energy-rich phosphorylated bonds per quantum. No attempt is made to explain why or how respiration is coupled with the hypothetical photochemical reaction.

curve is strictly linear and is measured bewteen a light intensity giving a photosynthetic rate approximately three times the rate of respiration and one giving a rate nine times the rate of respiration. If the curve, corrected for respiration, is extrapolated to zero intensity, it does not hit the zero point of gas exchange but rather a point about halfway between zero and the point at which respiration is compensated. Kok concludes that the slope of the curve at the very lowest light intensities must be twice the slope of the linear portion observed at higher intensities. Using only the latter part of the curve, he claims that the quantum yield of photosynthesis measured by him has the value 1/7.5 and, because that is greater than 1/8, he rejects theories based on the theoretical yield 1/8. (The experimental limit, of course, should be somewhat lower than the theoretical one.) We can discard this part of his conclusions because Rieke found an error in the photometry of Kok which makes the actual quantum yield more than 10% lower than the one calculated by this author. The corrected values are $\sim$ 1/9 and are, therefore, in agreement with the yield as measured by most observers, and half as high as Warburg's. In a few measurements made in an alkaline buffer he found that the rate curve had a slope 20% less steep than the one observed in the culture solution.

4. Connection Between the Hypothesis and Warburg's Observations of Cyanide Influence on Photosynthetic Rates in Chlorella

There are further observations which support the assumption that photochemical reduction of respiratory intermediates can occur in plants. The first, and most important, observation of this type was made by Warburg (9) in the year 1920 and interpreted by him by the very assumption we make use of in the present paper. He found that respiration in *Chlorella* is much less sensitive to cyanide than is photosynthesis. Concentrations of this poison, which slightly increase respiration, are able to suppress photosynthesis entirely. However, the gas exchange during illumination does not fall below the compensation point, even if much greater cyanide concentrations are used, which, as measurements during dark periods show, increase respiration considerably. Warburg's contention is the following: CO_2 has to undergo a chemical dark reaction to transform it into a substance which can be reduced photosynthetically. Cyanide in moderate concentrations poisons this preparatory reaction and, therefore, makes CO_2 photosynthesis impossible. Intermediates of respiration do not need such

preparatory dark reactions to become photochemically reducible. If they are caught by the photochemical reaction before being evolved as CO_2, the gas exchange of respiration is compensated and no pressure changes are observed in the light.

Van der Paauw (10) was the first one to become suspicious as to whether there was not a connection between Warburg's results concerning the influence of cyanide on algae and his determination of the quantum yield, but apparently Warburg did not regard it as likely. He probably saw clearly that a great difference in conditions exists between the two types of experiments. The reduction of respiratory intermediates may be the only photochemical reduction process occurring under conditions where photosynthetic CO_2 reduction is prevented, but that does not mean that the former process may be able to compete with the latter if the preparatory CO_2 fixation is able to proceed without hindrance. After all, photosynthesis can proceed approximately 20 times as fast as respiration and thus, at low light intensities, the concentration of the fixed CO_2 will be great compared with the concentration of respiratory intermediates. The effective concentration of the latter must be even smaller if one takes into account the fact that most of the respiration (perhaps all of it) takes place in parts of the cell outside of the chloroplasts, and that the diffusion of the respiratory intermediates into the chloroplasts may be slow.

Other observations are described in the literature which fit into the same pattern. Algae or leaves, irradiated under conditions where CO_2 is removed as efficiently as it can possibly be done, show that the gas exchange observed in the dark is fully compensated for in the light (11). The experiments indicate that, in this case, not only the intermediate products of respiration are photochemically reducible but also intermediate products of photoxidation.

The intermediates of respiration have a chance to be photochemically reduced only if they penetrate into the chloroplasts. According to the hypothesis put forward in the present essay, the premeability of the chloroplast membranes to these substances is not a normal property but rather one easily imposed on the membranes (especially in *Chlorella*) by suitable pretreatment of the plant material. This conclusion lends itself to experimental tests. For instance, it would be of interest to find out whether very young and diffuse cultures of *Chlorella*, not subjected to dense packing by strong centrifugation, will behave like Warburg's if irradiated in the presence of cyanide, or whether, in this case, cyanide

may be able to lower the gas exchange below the compensation point during exposure to light, because of the impermeability of the chloroplast membranes to the respiratory intermediates. To avoid damage by photoxidation, moderate light intensities should be used which, in the presence of cyanide, are sufficient to reach light saturation.

IV. Connection between the Main Hypothesis and Measurements of the Chlorophyll Fluorescence in Plants

1. *Respiration Intermediates are Preferentially Adsorbed at the Chlorophyll*

Our explanation is only possible if a strong preference exists for the reduction of respiratory intermediates. Experimental evidence indicates that the photosynthetic apparatus has indeed a special affinity for these substances. After Kautsky and coworkers (12) first observed that the chlorophyll fluorescence becomes several times stronger during the induction period than during the steady state of photosynthesis, much attention was paid to the general relation between fluorescence intensity and photosynthetic rates (12). Although no unanimity exists in respect to the interpretation, there is at least full agreement about the facts. The most important ones are the following: The fluorescence yield of the chlorophyll is always very low in plants. However, there exists a general relation between fluorescence intensity and the rate of photosynthetic oxygen production (of course, it must be recognized that quick changes in gas exchange cannot be recorded undistorted because of the slowness of the adjustment of gas equilibria between water and the atmosphere, whereas fluorescence intensities can be measured practically free of inertia of the recording instrument). If the fluorescence intensity becomes several times greater than normal, photosynthetic oxygen production becomes many times smaller. Measurements during the induction period clearly show the relationship. The fluorescence intensity shows a steep rise in the first seconds of irradiation followed by a slow transition to normal intensities in about one one minute. Correspondingly, the rate of oxygen production falls rapidly from the initial value to a deep minimum and rises in about a minute to its steady state. Sometimes with higher plants, and often with algae (especially *Chlorella*), the fluorescence intensity-time curve looks different. The decay after the first outburst is somewhat more rapid,

and a second broader wave of fluorescence intensity rise and fall develops. A corresponding time course is observed for the rate of oxygen production (13). The induction phenomena are more pronounced in old and dense algae cultures, and in leaves, than in suspensions of young algae. In some cases, very young and diffuse cultures of algae show no anomalies of the fluorescence (12) and no induction losses of the rates (5) at the beginning of the irradiation periods. However, anaerobic treatment of young algae causes induction phenomena to occur. If the chloroplast membranes are permeable due to age, density, prolonged anaerobicity, or other conditions, alkali can penetrate into the chloroplasts and the anomalies of the fluorescence intensity disappear entirely. It is, however, sufficient that alkali penetrates merely the outer cell membranes. This enhances the degree of ionization of the plant acids and thereby lowers their ability to permeate the chloroplast membrane. There is also a strong resemblance between the fluorescence curve and the one of CO_2 evolution *vs.* time as observed by Emerson during the first minutes of irradiation. The first high fluorescence maximum corresponds to the strong CO_2 outburst and the second lower fluorescence maximum corresponds to a second weaker outburst of CO_2.

Detailed explanations of these and connected phenomena are given elsewhere by the author and coworkers (12). A brief summary of the conclusions to which the theory leads follows: The cause of the induction phenomena is the inactivation of the oxygen-liberating enzyme by a metabolic excretion product of the algae which is water-soluble and which penetrates from the suspension into the chloroplasts. This poison is destroyed by oxidation, the photosynthetically produced peroxides being efficient oxidizing agents. The poison concentration in the cells is, therefore, greater during dark periods than during illumination, and consequently, at the start of an illumination period, the activity of the oxygen-liberating enzyme is low. Therefore, the photoperoxides are not removed quickly and will attack easily oxidizable substances such as carbohydrates. The first oxidation products of the carbohydrates are acids. These acids possess surface-active properties and displace CO_2 complexes and photosynthetic intermediates from the surface of the chlorophyll complexes. Just as surface-active narcotics, they increase the fluorescence intensity when they are adsorbed at the chlorophyll. The rapid rise in fluorescence intensity at the very start of the irradiation period is the result of the production of these

acids. The decay of the fluorescence to a steady state corresponds to a decreasing production of these acids because the oxygen-liberating enzyme becomes reactivated by removal of the poison by oxidation. The absence of the fluorescence anomalies in the presence of alkali indicates that the ionized acids are not strongly adsorbed at the chlorophyll or that they are unable to penetrate the chloroplast membranes.

2. Connection between Fluorescence Anomalies and the Emerson Effect, etc.

The time course of the Emerson effect coincides with that of the fluorescence intensity because the acids, which are abundant when the fluorescence yield is high, cause the liberation of CO_2 from the substance in which it is fixed preparatory to photosynthetic reduction.

In Warburg's algae the acid intermediates of respiration are already present at the start of the irradiation. The concentration of the preparatory fixation product of CO_2 should, therefore, be smaller and the rise of acidity connected with the induction phenomena will then give a smaller Emerson effect than the one occurring in Emerson's algae. If the surface-active acids can be made in the chloroplasts by an oxidation reaction between carbohydrates and photosynthetically produced peroxides, they should also appear under conditions where photoxidation occurs. That is indeed the case. When CO_2 is the limiting factor responsible for saturation, the fluorescence yield rises in the presence of sufficient oxygen to cause photoxidation. However, when only enough oxygen to sustain respiration is admitted, practically no rise of the fluorescence yield occurs because no acids are made by photoxidation. When oxygen is entirely absent, fermentation occurs and the acids produced by this process are able to penetrate to the photosynthetic apparatus because anaerobicity increases the chloroplast membrane permeability. As a result, the fluorescence yield is high at the very beginning of the irradiation. A rise in fluorescence intensity is also observed if, by prolonged irradiation, the carbohydrate concentration becomes high or if sugar is introduced into the leaves from outside. A high concentration of carbohydrates favors the reaction between peroxides and carbohydrates with the formation of surface-active acids, but the main reason for the rise in fluorescence intensity in this case seems to be the poisoning of the oxygen-liberating enzyme by a

metabolic product and the consequent increase in the peroxide concentration.

3. *Preferential Reduction of Respiration Intermediates*

All of the observations mentioned clearly indicate that partially oxidized carbohydrates containing one or more acid groups are strongly adsorbed at the chlorophyll complexes and are able to displace other substances from contact with the chlorophyll even if the latter are present in much higher concentration. This property of the acids is used by the plant to control photochemical activity and prevent photoxidation or excessive production of photosynthates. Thus, without exception, we observe that a rise of fluorescence intensity is connected with a decline in the photosynthetic rates. This seems to be in direct contradiction to the hypothesis on which our interpretation of Warburg's quantum yield measurement is based, namely, that such plant acids are preferentially reduced photochemically and, under certain conditions, are responsible for an increase in photochemical activity rather than for a decrease. However, the contradiction may be only an apparent one. The conclusion that 8 quanta are necessary to reduce one CO_2 molecule implies that two quanta are needed to split each water molecule. According to the kinetic theory of Franck and Herzfeld (14), this can be achieved if chlorophyll complexes activated by absorption of a light quantum serve as intermediate hydrogen donors and get back from water their hydrogen with the help of a second light absorption act. This means that the chlorophyll complexes have to react alternately with the substances to be reduced and with water. If the substances to be reduced are as strongly adsorbed as the intermediates of carbohydrate oxidation, this alternation will work efficiently only so long as their concentration is small. If these intermediates are produced by photoxidation or by reaction between carbohydrates and photosynthetic peroxides, their concentration will be large and they will cover both the hydrogenated and the dehydrogenated chlorophyll complexes and prevent the rehydrogenation of the latter. As a result, all photochemical activity will stop. According to Clendenning and Gorham (15), the rate of quinone reduction by illuminated chloroplasts first rises and then quickly declines as the quinone concentration is increased, even under conditions where light intensity is the limiting factor. The interpretation may be a similar one; a substance may react photochemically at low concentrations and act as a narcotic at higher ones.

The complete cessation of all photochemical activity when enough partially oxidized carbohydrate is adsorbed on the chlorophyll complexes is not the only possible explanation of the changes in fluorescence intensity and the regulation of the photosynthetic output of oxygen. The same result can be achieved if the partially oxidized molecules are reduced photochemically and then immediately reformed by oxidation. The same limited amounts of material can thus be used over and over again, and the photosynthetic apparatus will be transferred from a state in which it does work to a state resembling that of an idling engine. Suitable experiments may make possible a decision between the two explanations, but the writer has thus far been unable to propose the right kind of experiments.

V. The Reason for the Conflicting Results in the Determination of Photosynthetic Intermediates May Be the Same as the One Responsible for the Contradictions in Quantum Yield Measurements

Finally, a few remarks may be added supplementing those made in the introduction about the similarity between the contradictions encountered in the attempts to identify intermediates of photosynthesis and the ones discussed above in the interpretation of the measurements of photosynthetic quantum yield. Calvin and coworkers (16) came to the conclusion that substances known to be intermediates of respiration act also as intermediates of photosynthesis. Gaffron, Fager and Brown (16), on the other hand, find entirely different properties in the substance or substances which, according to their results, must be an intermediate of photosynthesis. Fager, who has not fully identified the chemical nature of this substance, presents chemical evidence excluding all the substances mentioned by Calvin and coworkers. The Chicago group stated at the meeting of the Am. Assoc. Advancement Sci., in December, 1947, that the observations made in California seem to be related to respiration (and fermentation) of the plants. The present writer is in agreement with that statement but believes that all interactions between respiration and photosynthesis must also be taken into account. Such influences will occur if the chloroplast membranes of the plants used in California become permeable to respiration intermediates. Indeed the pH of 4.5–5 used in the California experiments is favorable for membrane permeation of plant acids, while pH 7.8 used in Chicago will prevent that process.

Summary

Warburg's observation, that the quantum yield of photosynthesis is $\sim 1/4$, deviating from that of others by a factor of two, can be explained by the assumption that, under the particular conditions of his experiments, a photochemical reduction of intermediate respiration products rather than CO_2 reduction is observed.

The condition necessary for the replacement of normal photosynthesis by this other process seems to be an abnormal permeability of the chloroplast membranes. The consequences of this hypothesis have been compared with experimental evidence.

Indications are mentioned for the assumption that the contradictions in the results gained so far on intermediates of photosynthesis may be caused by the same variability of the permeability of membranes which is supposed to be responsible for Warburg's results on quantum yields.

Note Added in Proof

Since this paper was written, O. Warburg, D. Burk, V. Schocken and S. Hendricks have carried out new experiments on photosynthetic quantum yield and have reported their findings at a meeting of the Society of General Physiologists, at Woods Hole, on June 22, 1949.

The evaluation of these data must wait until they are published *in extenso*. However two sets of experiments have been presented which seem to make it necessary to change one of the main assumptions used in the present paper. They are:

(a) Quantum yield measurements with the Warburg method gave again a yield of 1/4 provided the suspension was quite acid (pH $\sim$ 4–5); higher pH values gave smaller yields approaching the yield of $\sim 1/10$. The measurements have been extended well into the region above the compensation point. Since the narrow beam of red monochromatic light (from the monochromator) was not intense enough to overcompensate several times the respiration of all the algae (only about 1/10 to 1/20 of the algae in the vessel were exposed simultaneously to the light), the beam was superimposed on a general irradiation of the vessel with white light of unknown absolute intensity. The white light, though of considerably weaker intensity than the red beam, illuminated a larger portion of the algae, and was adjusted to give gas exchanges about 3 times those of respiration. The quantum efficiency of the red light remained unaltered by irradiation with the white light.

(b) When the CO_2 concentration in the vessel was kept exceedingly low, illumination with the red light beam did not measurably change the oxygen consumption of normal respiration.

It is difficult (though not impossible) to reconcile the hypothesis that reduction of half-oxidized respiration products is responsible for Warburg's high quantum yield, with the fact (a) that it remains high even when the total photosynthetic activity becomes several times higher than respiration. Moreover, the fact mentioned under

heading (b) is in direct contradiction to that hypothesis, which therefore fails to give a satisfactory explanation of Warburg's results. However, the present author is still convinced that the above discussion contains a good deal of material useful for the reconciliation of the differences in the results of quantum yields and of the chemical nature of intermediates of photosynthesis. He believes that there are two different photosynthetic processes, one with the quantum yield of 1/4, the other with 8 quanta and that the former is exceptional, taking place only when the chloroplast membranes become permeable, thus permitting mutual interactions between photosynthesis and respiration. High acidity, which discharges acids, is obviously one of the conditions necessary for the occurrence of that process.

If, as the new experiments indicate, the replacement of CO_2 reduction by that of half-oxidized respiration products is not responsible for the higher quantum yield process observed by Warburg, it might be that a part of *the energy* of respiration can be used for photosynthetic processes. It is often assumed that energy-rich phosphate bonds are made by the light and that their energy is used to reduce CO_2 in dark reactions similar to the synthesis occurring as a by-product of dark respiration. We have many reasons to reject this idea. However, it might be possible that the energy stored in the phosphate bonds produced by respiration might be transferred to phosphate bonds of the CO_2 complex and of intermediate products of photosynthesis. In that way, the energy of 12 K-cal. would be available in the molecules to be reduced before each photochemical reaction and, with that additional energy, photosynthesis may proceed with 4 quanta. However, this photosynthesis could, even if all other conditions are favorable to it, only proceed to a maximum rate of 1.5 times that of respiration. Any photosynthesis in algae beyond 1.5 times respiration would need 8 quanta.

If we want to explain by that hypothesis some of Warburg's new results mentioned under (a) we must introduce the assumption that, in Warburg's special arrangements, the algae exposed to the strong but short-lasting illumination of the red beam are in a better position to make use of the energy of the phosphate bonds for photosynthesis than those algae exposed for a longer time to the weaker intensity of the white light. It is possible to give kinetic reasons for such behavior, but this would be premature as long as insufficient experimental evidence is available in support of the main hypothesis.

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Transformation of Tryptophan to Nicotinic Acid Investigated with Delayed Supplementation of Tryptophan [1]

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Introduction

Since the experiments of Krehl, Tepley and Elvehjem (1) on the curative effect of tryptophan in experimental niacin deficiency, several authors have reported that the excretion of nicotinic acid is increased more after the administration of extra tryptophan in the form of the pure amino acid than in the form of extra casein (2). This finding suggests that tryptophan may also be utilized in a way which is independent of protein synthesis.

It has been shown in earlier experiments (3,4) that tryptophan, fed as a delayed supplement to rats on tryptophan-free amino acid mixtures, neither promotes growth nor prevents cataract formation. It was, therefore, assumed that the same technique might be used to determine whether or not the participation of tryptophan in niacin synthesis occurs independently of protein synthesis.

It was further intended to investigate whether vitamin B_6 is specifically involved in the transformation of tryptophan (5), or whether the absence of this essential nutrient interferes in a non-specific way with the nicotinic acid production. Since there are no indications that the oil-soluble vitamins A and D present in cod-liver oil are directly involved in the amino acid metabolism, we investigated the effect on nicotinic acid formation of a diet containing all the necessary growth-factors with the exception of cod-liver oil.

[1] This work has been supported in part by a research grant from the National Vitamin Foundation.

[2] One part of the experiments was submitted by H. D. Gatchell in partial fulfillment of the requirements for the degree of Master of Science in the Department of Physiology, School of Medicine, University of Southern California.

Methods

Infantile male Sprague-Dawley rats of 40–45 g. body weight were placed in individual cages provided with wire mesh bottoms. The body weight of the animals and the food consumption were determined daily.

The rats had access to their diets from 4 P.M. until 7 A.M. the next day and the supplement was administered by dropper at 12 M. The tryptophan solution was prepared by dissolving 75 mg. of L-tryptophan in 25 ml. of hot 2% gelatin solution.

TABLE I

Composition of Diets

Component	Diet number					
	I	II	III	IV	V	VI
1. Basal diet[a]	86 g.	86 g.	86 g.	86 g.	86 g.	86 g.
2. Vitamin-free casein	9 g.	9 g.	9 g.	10 g.	10 g.	10 g.
3. Tryptophan-free Casein acid hydrolyzate	—	—	—	3 g.	3 g.	3 g.
4. Gelatin	6 g.	6 g.	6 g.	—	—	—
5. L-Tryptophan	—	—	150 mg.	—	—	100 mg.
6. Nicotinic acid	—	20 mg.	—	—	—	—
7. Cod liver oil	2×wk. 2 drops	2×wk. 2 drops	2×wk. 2 drops	2×wk. 2 drops	—	—

[a] Basal diet: 820 g. sucrose, 30 g. corn oil, 40 g. U. S. P. salt mix, 4.0 mg. thiamin, 6.0 mg. riboflavin, 5.0 mg. pyridoxine, 40 mg. calcium panthothenate, 1 g. choline, 2.0 mg. vitamin K, 200 mg. inositol, 0.2 mg. biotin, 1.0 mg. folic acid, 1.6 g. cystine.

This solution could be cooled to about 30°C. without precipitation of the amino acid. The niacin solution contained 10 mg. niacin in 2.5 ml. 2% aqueous gelatin solution. The animals not receiving supplement were given at the same time equivalent amounts of a 2% gelatin solution. For the composition of the diets see Table I.

The nicotinic acid excreted in the urine was determined by the modified colorimetric method of the Research Corporation (6).

Experimental

A. In the first group of experiments the rats were placed on a deficient diet No. I. (7). After a depletion period of 6 days, during which the rats did not gain weight, the animals were divided into 5 groups, each group containing 4 rats. The condensed results are presented in Table II.

The rats of Gp. 1, continued on Diet I, did not gain more than the animals of Krehl *et al.* (7) on a similar deficient diet. In Gp. 2, the addition of nicotinic acid improved the growth considerably. In Gp. 3, a diet, supplemented with tryptophan, resulted in a similar improvement of growth. In Gp. 4, the rats received the deficient Diet I during the night and at 12 M., as a delayed supplement, 0.5 ml. of the niacin solution. In Gp. 5 the rats were kept on deficient Diet I for the night and received as a delayed supplement at 12 M, 0.5 ml. of the tryptophan solution.

TABLE II

Average Growth of Rats with Delayed Supplementation of Tryptophan for 21 Days

Group no.[a]	Diet (see Table I)	Supplement	Average group gain per day	Average individual gain per day	Average group dietary intake per day	Ratio of dietary intake over weight gain
			g.	*g.*	*g.*	
1	I	—	1.2	1.7 1.2 0.9 1.2	6	5
2	II	—	2.2	1.9 2.4 2.3 2.4	7	3.2
3	III	—	2.4	3.3 2.0 2.3 2.0	8	3.3
4	I	Niacin	2.0	3.2 2.4 2.5 2.5	8	3.2
5	I	Tryptophan	2.5	2.0 2.0 2.0 1.8	7	3.5

[a] Each group contained 4 rats.

Comparing the growth of the Gps. 2 and 3 with that of 4 and 5 shows that *nicotinic acid and tryptophan fed as a delayed supplement promotes growth as well as when fed simultaneously with the deficient diet*

B. In another group of experiments we investigated whether the *delayed* supplementation of tryptophan also increased the nicotinic acid excretion. In these experiments, the rats were depleted 14 days on an imbalanced diet, No. IV. After this depletion period, Gp. 6 received the same imbalanced diet daily from 4 P. M. until

7 A. M. and no supplement. Gp. 7 received an imbalanced diet at night and at 11 A. M. a supplement of 20 mg. tryptophan.

Table III shows that, in both groups, nicotinic acid excretion decreased during the depletion period and continued to be low in Gp. 6. *In Gp. 7, which received tryptophan as a delayed supplement, however, with the resumption of growth there was a considerable increase in nicotinic acid excretion.*

C. In a third group of experiments we investigated rats which did not receive cod liver oil in their diet. These rats were placed on an imbalanced Diet V for 7 days. After this period, the rats were divided into 2 groups. Gp. 8 received, from 4 P. M. to 7 A. M., Diet VI containing tryptophan. The rats of Gp. 9 received the imbalanced diet from 4 P. M. until 7 A. M., and at 11 A. M. tryptophan as a delayed supplement.

During the depletion period the average nicotinic acid excretion in both groups dropped from 147 γ and 161γ/day to 31γ and 18γ/day for Gps. 8 and 9, respectively. Growth also declined to about 1 g. per rat per day.

During the next 11 days the growth and the nicotinic acid excretion increased slightly in both groups. At the end of this period the symptoms of vitamin A deficiency, such as ophthalmy, became apparent and, at the same time, growth and nicotinic acid excretion decreased in both groups to a very low value. At this time the animals did not show any signs of vitamin D depletion.

On the 19th day, cod liver oil was administered and, with the disappearance of symptoms of vitamin A deficiency, rapid growth started, paralleled by a considerable increase in nicotinic acid excretion.

These experiments show that *omission of cod liver oil from the diet interferes not only with growth but also with urinary nicotinic acid excretion, and that, after the addition*

TABLE III

Nicotinic Acid Excretion of Rats on Delayed Supplementation of Tryptophan

Rat group no.[a]	Duration of period	Diet (see Table I)	Daily av. gain per rat	Daily av. food intake per rat	Av. nicotinic acid excretion in urine
	days		*g.*		
		A. Depletion period			
6	14	IV	0.3	3.9	48
7	14	IV	0.4	3.5	65
		B. Test period			
6	6	IV	−0.14	3.5	55
7	6	IV and delayed supplementation of tryptophan	1.8	5.5	161

[a] Each group contained four rats.

TABLE IV

Nicotinic Acid Excretion of Vitamin A and D Depleted Rats

Rat group no.[b]	Duration of period	Diet (see Table I)	Supplementation with tryptophan	Daily av. gain per rat	Daily av. food intake per rat	Av. nicotinic acid excretion in urine
	days			*g.*	*g.*	γ
A. Depletion period						
8	7	V	---	1.0	6.5	(141)[a] 31
9	7	V	-	1.0	6.3	(161)[a] 18
B. Tryptophan supplementation period						
8	19	VI	Mixed in the diet	0.85	6.8	40
9	19	V	Delayed supplement	0.7	6.2	36
C. Cod liver oil and tryptophan supplementation						
8	13	VI	Tryptophan mixed in diet	2.5	9	106
9	13	V	Delayed supplementation	2.8	8	148

[a] Value on first day of depletion period.
[b] Each group contained four rats.

of cod liver oil, growth and nicotinic acid excretion are increased nearly equally, independently of whether the tryptophan is mixed in the diet or fed as a delayed supplement.

Discussion

These experiments indicate that tryptophan given as a delayed supplement to niacin-deficient rats increases growth, and augments the urinary excretion of nicotinic acid. It was shown earlier that feeding of delayed supplements of tryptophan to animals kept on tryptophan-free diets, does not promote growth (3) and does not prevent cataract formation (4). Therefore, the corrective effect of tryptophan in niacin deficiency seems to be exerted independently of protein synthesis by direct transformation of this amino acid into nicotinic acid.

It should be pointed out that the animals in the present experiments grew, even though tryptophan was given as a delayed supplement. This seems to be in contradiction to earlier experiments, in which a totally

tryptophan-free diet was supplemented. In the present experiments, however, the deficient diet *per se* contains enough tryptophan, in the form of casein, to promote growth, and only the harmful effect of the added imbalanced amino acid mixture had to be corrected by supplementation of tryptophan.

These experiments show, secondly, that omission of cod liver oil from the diet interferes with the growth-promoting effect of tryptophan. There was a possibility that in such animals where growth, *i.e.*, apposition of newly formed body protein, ceases, a larger amount of tryptophan may be transformed to niacin. The experiments, however, show that deficiency of vitamins, normally supplied with cod liver oil, interferes with the transformation of tryptophan to niacin in the same way as does vitamin B_6 deficiency.

Since the completion of these experiments, a paper by Junqueira and Schweigert (5) appeared showing that, besides vitamin B_6 deficiency, a deficiency of B_1, B_2, pantothenic acid, or folic acid decreases the transformation of tryptophan to nicotinic acid. Our results, and the results of the above authors, suggest that Vitamin B_6 may not specifically participate in the transformation of tryptophan to nicotinic acid. It seems more probable that any deficiency in essential food elements may interfere with growth promotion and with the transformation of tryptophan to nicotinic acid.

Summary

1. Tryptophan given as a delayed supplement to niacin-deficient rats increases growth and augments urinary excretion of nicotinic acid, as well as when fed simultaneously with the deficient diet.

2. Omission of cod liver oil from the diet interferes with the growth-promoting effect of supplementary tryptophan and inhibits the transformation of this amino acid to niacin.

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Increase in Yeast Respiration in Presence of Several Steroids and Diethylstilbestrol

While studying the effects of steroids on enzyme systems of yeast, it was observed that several steroids and diethylstilbestrol produced a marked increase in the endogenous respiration of yeast. When respiration was measured in the presence of glucose, on the other hand, a diminished respiration was observed following addition of several of the compounds.

It is seen from the tables that the inhibitory effect of diethylstilbestrol, desoxycorticosterone, and testosterone on respiration with added glucose is in inverse order to their effect on acceleration of the endogenous respiration of yeast. Inhibition of respiration by rat brain

TABLE I

Accelerative Effects on Endogenous Respiration[a]

Substance added[b]	Oxygen uptake, μl.			
	30 min.	60 min.	90 min.	120 min.
Water (control)	14	24	34	41
Diethylstilbestrol	114	274	345	384
Cholesterol	62	87	103	114
Desoxycorticosterone	34	63	87	106
Testosterone	18	35	52	68
Progesterone	14	26	38	48

[a] Four experiments in duplicate on yeast from two sources were carried out. The table shows typical average results from one experiment.

[b] Warburg vessels contained 7.7 mg. (dry weight) of washed bakers' yeast and phosphate buffer (0.066 *M*), pH 6.8. Equilibration was carried out for 2 hr. before tipping in the substances from side arms, in order to reduce endogenous metabolism to a low, relatively constant level. Two mg. of the steroids and of stilbestrol were added as finely ground suspensions in water at zero time. Gas phase, air; temperature, 37°C.; 10% KOH in the central well.

homogenates in the presence of several steroids has recently been reported (1), while Verzar (2) has demonstrated that desoxycorticosterone accelerates glycogenolysis and inhibits glycogen synthesis in rat diaphragm.

It would appear, therefore, that the steroids and diethylstilbestrol diminish yeast respiration with added glucose by inhibiting glucose utilization. When no exogenous substrate is added, yeast respiration is probably due primarily to utilization of endogenous glycogen, and the rate of respiration is probably limited by the rate of glycogenolysis. Since several steroids and diethylstilbestrol markedly increased the endogenous respiration of yeast, it is suggested that these substances produce their effect by accelerating glycogenolysis.

TABLE II

Inhibitory Effects on Respiration with Added Glucose[a]

Substance added[b]	Oxygen uptake, μl.			Per cent inhibition	Per cent inhibition rat brain homogenates (Data of Gordan (1))
	30 min.	60 min.	90 min.		
Water (control)	50	101	148	—	–
Diethylstilbestrol	27	38	44	70	91
Cholesterol	54	105	154	-	14
Desoxycorticosterone	33	66	98	34	87
Testosterone	41	84	131	11	24
Progesterone	47	98	146	1	28

[a] Two experiments in duplicate were carried out. The table shows typical average results from one experiment.

[b] Warburg vessels contained 0.77 mg. (dry weight) of washed yeast, phosphate buffer (0.066 M) pH 6.8, and 0.2% glucose. Respiration was measured for 1 hr. before the substances (2 mg.) were tipped in from side arms. Data are uptakes following tipping in of side arm contents. Conditions otherwise as in Table I.

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Inhibition of Ergostanyl Acetate by 7-Dehydrocholesteryl Bromide

The activity of ergostanyl acetate in curing the stiffness syndrome in guinea pigs has been reported from this laboratory (1) and by Petering and coworkers (2).

We wish to report here the results of some tests made on various combinations of sterols in a search for synergisms and antagonisms in this class of compounds, as measured by the guinea pig assay for the anti-stiffness factor.

Weanling pigs of the Hartley strain were placed on the diet previously described (1), and allowed to deplete for approximately a week, when most of the animals developed a 3 + stiffness. A curative test of 5–7 days was then employed.

TABLE I

Antagonism of Ergostanyl Acetate by 7-Dehydrocholesteryl Bromide

Supplement	Test level	No. of assays	Total no. animals	Animals showing curative response
				per cent
1 None	—	5	38	2
2 Ergostanyl acetate	10 γ	5	38	63
3 Ergostanyl acetate	200 γ	1	8	69
4 As 2+7-dehydrocholesteryl bromide	6 mg.	3	22	0
5 As 2+7-dehydrocholesteryl bromide	3 mg.	2	15	0
6 As 2+7-dehydrocholesteryl bromide	1 mg.	1	7	36
7 As 2+7-dehydrocholesteryl bromide	0.6 mg.	1	8	31
8 As 2+7-dehydrocholesteryl bromide	6 mg.	1	7	64
9 As 3+7-dehydrocholesteryl bromide	3 mg.	1	7	64
10 As 2+7-dehydrocholesterol	6 mg.	1	8	50

All of the supplements were dissolved in butyl succinate and injected intramuscularly each day. The 7-dehydrocholesteryl bromide was prepared by the method of Bernstein *et al.* (3) and the ergostanyl acetate by standard procedures (4).

The results of 5 separate experiments are combined in Table I. The expected responses were obtained from 10γ daily of ergostanyl acetate. Of a number of compounds tested, 7-dehydrocholesteryl bromide was the most active as an inhibitor. Levels of 3 mg. or more per day of this

compound completely inhibited the response of 10γ of ergostanyl acetate. This inhibition faded out at levels below 3 mg. per day. The antagonism was completely reversed by 200 γ daily of ergostanyl acetate, indicating ready reversibility.

Animals receiving this inhibitor became more severely deficient by the end of the test period than those in the negative control group. However, this effect with other diets has not been tested. Thus 7-dehydrocholesteryl bromide appears to be a reversible antagonist of the anti-stiffness factor. The existence of a naturally occuring antagonist has been reported by van Wagtendonk and Wulzen (5).

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Effect of Vitamin B_{12}, Animal Protein Factor and Soil for Pig Growth

Vitamin B_{12},[1] an animal protein factor supplement (Lederle),[2] and soil[3] were added to a control ration of ground yellow corn 57, peanut meal 41.5, bone meal 0.5, limestone 0.5, salt 0.5%, plus vitamins A and D at levels of 5000 I. U. and 700 I. U., respectively, per pig daily. The other vitamins were added at the following levels/100 lbs. of feed: thiamine 1 g., riboflavin 230 mg., niacin 2.33 g., pantothenic acid 1 g., pyridoxine 375 mg., choline 19.4 g., and folic acid 22.7 mg. The control ration contained all the known vitamins which the pig has been shown to need (1). The first trial lasted 6 weeks and the second trial for 5 weeks.

[1] Vitamin B_{12} concentrate (charcoal) from Dr. D. F. Green, Merck & Company, Rahway, New Jersey. (Contained 2 mg. of B_{12} activity/lb.)

[2] Animal protein factor supplement (fermentation product) (N195 and N199B) from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, New York.

[3] Top 3 inches of soil, strained of vegetable matter and dried at room temperature.

The addition of 5% soil to the basal ration was beneficial, indicating that soil was supplying some unknown factor(s). This is in agreement with previous work (2), when 5% soil added to a purified ration containing all known vitamins needed by the pig also supplied an unknown factor(s).

In Exp. 1 the addition of the animal protein factor supplement to the basal ration increased the rate of gain 26%. In Expt. 2, where much smaller pigs were used, the animal protein factor supplement resulted in approximately 2.5 times the gain of that obtained on the basal ration.

In Expts. 1 and 2, the addition of vitamin B_{12} concentrate to the basal ration was of no apparent benefit. In the third week of the second experiment, the level of B_{12} concentrate was doubled. The pigs decreased in rate of gain when the level of B_{12} was increased, thus showing no benefit for increased levels of B_{12}.

TABLE I

Expt. no.	No. pigs	Av. starting weight	Ration fed	Av. daily gain	Hb av.
		lbs.		*lbs.*	
1	4	33.3	Basal	1.14	13.7
	4	32.5	Basal+1.1% animal protein factor supplement	1.44	15.2
	4	33.0	Basal+0.2% vitamin B_{12} concentrate	1.21	13.3
	4	33.3	Basal+5% soil	1.34	12.4
2	6	18.3	Basal	0.29	
	6	18.1	Basal+2.2% animal protein factor supplement	0.73	
	5	18.5	Basal+0.4% vitamin B_{12} concentrate	0.25	

Catron and Culbertson (3) found that B_{12} was of benefit when added to a corn soybean oil meal, alfalfa, mineral ration to which vitamins A and D were added. Why the results obtained in this experiment are different, it is difficult to state. It is possible that, since we used peanut meal instead of soybean oil meal, it may make a difference. In addition, in these experiments, alfalfa meal was not included, whereas all B-complex vitamins which the pig has been shown to need (1) were added to the control ration, thus, possibly, accounting for the different results. It is possible that the B_{12} charcoal concentrate used did not have any activity, although this in not likely to be the case. It is also possible that vitamin B_{12} is only one of the factors in the animal protein factor

supplement and that the other factor(s) must be present before vitamin B_{12} will be of benefit.

The addition of the APF supplement seemed to stimulate hemoglobin formation; however, data are needed with more pigs before this can definitely be stated.

These data show that the addition of the animal protein factor supplement was of considerable benefit, especially with lighter hogs, when added to the basal ration, whereas the vitamin B_{12} concentrate was of no apparent help. These data show that, under the conditions of this experiment, the animal protein factor supplement of Lederle Laboratories and the vitamin B_{12} charcoal concentrate were different in their response, and that the animal protein factor supplement supplies an unknown factor or factors for the young pig fed a corn-peanut meal ration. Discussion is given as to possible reasons why the B_{12} charcoal concentrate was of no benefit.

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On the Probable Identity of Several Unidentified Growth Factors [1]

In 1946, Guirard, Snell and Williams (1) described an assay method for a naturally-occurring, water-soluble substance which duplicated the growth-promoting effect of acetate for *Lactobacillus casei*. Preparations concentrated approximately 40-fold from yeast extract were 400 times as active, on the weight basis, as acetate in promoting growth. On

[1] Supported in part by grants from the Schenley Research Foundation and from Parke, Davis and Co. We are indebted to Dr. I. C. Gunsalus and to Drs. T. H. Jukes and E. L. R. Stokstad for samples of the concentrates referred to in the text.

these grounds it appears logical to ascribe a catalytic role in the production of acetate to the active factor in such concentrates.

These considerations, and certain similarities in stability and distribution, led us to test concentrates (a) of an unidentified factor described by O'Kane and Gunsalus (2) that is required by resting cells of *Streptococcus faecalis* for oxidation of pyruvic acid, and (b) of "protogen," an essential growth-factor for *Tetrahymena geleii* described by Stokstad *et al.* (3). Highly purified preparations of each of these substances were extremely active in promoting growth of *Lactobacillus casei* in the absence of acetate (Table I) under previously described conditions (1). Concentrates of the pyruvate oxidation factor with relative approximate purities of 1, 70, and 3000 units/mg. by enzymatic assay (2) had relative activities of approximately 1, 90, and 3300 for *L. casei*. A preparation of "protogen" purified 10,000 times over a standard liver preparation by *Tetrahymena* assay, proved extremely active for *L. casei*; 3 mγ of this preparation sufficed to duplicate the growth-promoting activity of 400 γ of sodium acetate. The amounts of the latter concentrate required by *L. casei* for growth are

TABLE I

Comparative Activities of Acetate, Pyruvate Oxidation Factor, and Protogen in Promoting Growth of Lactobacillus casei

Sodium acetate		Yeast extract		Pyruvate oxidation factor[b]		Protogen[c]	
γ/10 ml.	Turbidity[a]	γ/10 ml.	Turbidity[a]	mγ/10 ml.	Turbidity[a]	mγ/10 ml.	Turbidity[a]
0	94	0	94	0	94	0	94
100	76	30	64	1	88	0.3	89
300	62	100	53	3	76	1.0	67
500	56	300	47	10	66	3.0	60
1,000	50	1,000	42	30	59	10.0	45
3,000	40	10,000	32	100	49	30.0	43
10,000	37			300	46		

[a] Per cent of incident light transmitted, uninoculated medium = 100. Incubated 30 hr. The medium and procedure were those described by Guirard, Snell and Williams (1).

[b] From Dr. I. C. Gunsalus; purified approximately 3000 times over yeast extract.

[c] From Dr. T. H. Jukes; purified approximately 10,000 times over a standard liver preparation.

quantitatively similar to the amounts of pyridoxal or folic acid required for growth of this organism.

From these results it appears highly probable that protogen, the pyruvate oxidation factor, and the acetate-replacing factor for *L. casei* are identical. It has been indicated (3) that more than a single form of this factor occurs naturally. Unpublished data (4) show that this factor is also one of the substances which promotes rapid growth of *Streptococcus faecalis* from small inocula in acetate-free media (*e.g.*, 5,6). In confirmation of previous data (1,7), and in marked contrast to their effect on *L. casei*, concentrates of pyruvate oxidation factor and of protogen were relatively ineffective in replacing acetate for *Lactobacillus arabinosus.*

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Effect of APF Supplement on Pigs Fed Different Protein Supplements

Cunha *et al.* (1) showed that an APF supplement (Lederle Laboratories) was of considerable benefit and supplied an unknown factor(s) when added to a corn-peanut meal ration (same ration as fed in Lot 1), whereas a B_{12} charcoal concentrate (Merck & Co.) was of no benefit when added to the same ration. In this experiment, peanut meal, soybean oil meal and fish meal made up 41.5, 44.5, and 30%, respectively, of the ration. All rations contained the same amount of crude protein which was 22.78 (± 0.05%). The remainder of the ration in each lot consisted of corn, bone meal 0.5, limestone 0.5 and salt 0.5%. Pigs in all lots received vitamins A and D at levels of 5000 I. U. and 700 I. U., respectively, daily. The other vitamins were added in all rations at the following levels/100 lbs. of feed: thiamine 1 g., riboflavin 230 mg., niacin 2.33 g., pantothenic acid 1 g., pyridoxine 375 mg., choline 19.4 g., and folic acid

22.7 mg. Thus, all the known vitamins which the pig has been shown to need (2) were added to all the rations fed. The trial lasted for 37 days. Five pigs were used in each lot.

The addition of the APF supplement to the corn-peanut meal ration resulted in 2.13 times the rate of gain obtained on the control ration. This is in agreement with previous work by Cunha *et al.* (1), where the addition of the APF supplement, at the same level, to the same corn-peanut meal ration (Lot 1) increased the rate of gain approximately 2.5 times.

TABLE I

Lot no.	Av. starting weight	Protein supplement fed	Av. daily gain
	lb		*lb.*
1	27.1	Peanut meal	0.62
2	27.1	Peanut meal + APF supplement[a]	1.40
3	26.4	Soybean oil meal	1.01
4	27.0	Soybean oil meal + APF supplement[a]	1.31
5	27.9	Fish meal[b]	1.29
6	27.8	Fish meal + APF supplement[a]	1.45

[a] Animal Protein Factor supplement (fermentation product) obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, New York.

[b] Fish meal, 60.2% protein, obtained from Ralston Purina Co., St. Louis, Missouri, and which satisfied their standards for being of very high quality.

The addition of the APF supplement to the corn-soybean oil meal ration increased the rate of gain approximately 30%. These data show that the APF supplement is much more beneficial when added to a corn ration containing peanut meal than when it contains soybean oil meal.

The addition of the APF supplement to the corn-fish meal ration was beneficial to a small extent. This may mean that fish meal, or particularly the fish meal used, is not quite high enough in the factor or factors which the APF supplement supplies.

Of much interest is the finding that the addition of the APF supplement to a corn-peanut meal ration and to a corn-soybean oil meal ration resulted in gains being obtained similar to those on a corn-fish meal ration (Lot 5). This shows that the APF supplement supplied an unknown factor or factors which caused peanut meal and soybean oil meal to come up to the fish meal used in feeding value for the pig. When the APF supplement was added to the peanut meal ration

(Lot 2) the results obtained were about as good as when it was added to fish meal ration (Lot 6).

These data indicate that the APF supplement supplies an unknown factor or factors for the pig, and that it increased the feeding value of peanut meal and soybean oil meal so that these plant protein supplements were similar in feeding value to the fish meal used.

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Does Light Inhibit the Respiration of Green Cells?

It is well known that the oxygen consumption of green cells may be decreased by illumination. The light intensity at which the oxygen exchange of a given culture becomes zero may be termed "compensating." Above this light intensity positive evolution of oxygen gas is observed. The mechanism of compensation by light has until now remained uncertain, but the simplest explanation is obviously the production of oxygen gas by photosynthesis; that is, since light clearly produces oxygen gas above compensation it is reasonable to suppose that it also does so below compensation. However, the idea, old as the science of photosynthesis, still persists that light inhibits respiration *per se*, either anticatalytically (as by inactivating respiratory enzymes), or by reducing intermediates of respiration. If this idea were true, most computations of photosynthetic efficiency would be invalidated because they have been carried out below the compensation point.

A decision on this much-discussed problem has been obtained from the following type of experimentation, performed many times. In the main compartment of a rectangular vessel, with two side-arms containing alkali, was placed a suspension of *Chlorella pyrenoidosa* cells in acid culture medium (pH 4.8), with air as gas phase. The vessel,

attached to a manometer, was rapidly shaken and alternately darkened and illuminated from below with a beam of completely absorbed red light of the *same* intensity ($\sim$0.25 microeinsteins/min./3 cm.^{2} area) as employed in simultaneous quantum efficiency determinations on aliquot suspensions by the 2-vessel method with 5% CO_2 in air as gas phase. In the vessel with low CO_2 pressure, negligible light action was observed, the oxygen consumption in the dark and in the light being practically identical, whereas, with aliquots of the same suspension under otherwise identical conditions except for adequate CO_2 pressure, high efficiencies of 3 to 5 quanta absorbed per molecule of O_2 produced were observed both below and well above the compensation point, with no change in dark respiration at the widely different CO_2 pressures involved (see example).

This absence of light action on respiration at low CO_2 pressures may appear to contradict the experience of other investigators who, since the use of manometry in photosynthesis, have observed, in vessels containing alkali in side-arms or middle compartments, that the respiration of Chlorella could be compensated by light. The explanation for this apparent discrepancy is that such experiments were carried out with too high light intensities that were not controlled quantitatively by means of simultaneous efficiency determinations. Light and alkali compete for the small amount of CO_2 formed in respiration, so that, for every CO_2 pressure, however low, a light intensity exists that will compensate respiration, as we have confirmed when we have used light of sufficiently high intensity.

Our experiments show conclusively that red light does not inhibit the respiration *per se* when light intensities are employed that yield high photosynthetic efficiencies. When light compensates respiration it does so by the independent process of photosynthesis, the gas exchange of which happens to be the opposite of that of respiration. This result has been confirmed in a different way by determination and comparison of photosynthetic efficiency below and above the compensation point. Under the conditions of our experiments, reported in detail elsewhere[1], the same high quantum efficiencies of 3–5 quanta per O_2 are obtained up to intensities at least five times the compensating intensity. That is, one molecule of oxygen developed above the compensation point, or

[1] In press, *Science*, and *Biochim. Biophys. Acta* (Meyerhof Festschrift, October, 1949); and report delivered at meeting of Society of General Physiologists, Woods Hole, Mass., June 22, 1949.

one less molecule of oxygen consumed below the compensation point, as the result of light action, represents the same gain in chemical energy. All theories of light action should be in harmony with this now-established thermodynamic fact.

Example

Each of three vessels contained 230 mm.^{3} aliquots of *Chlorella pyrenoidosa* cells suspended in 7 cc. of culture medium (5 g. $MgSO_4 \cdot 7H_2O$, 2.5 g. KNO_3, 2.5 g. KH_2PO_4, 2 g. NaCl, and 5 mg. $FeSO_4 \cdot 7H_2O$ in 1 l. of filtered, unsterilized well water at pH 4.5–5). Temperature, 20°C. Horizontal shaking at the rate of 150 cycles/min. at 2 cm. amplitude. Total intensity of red light beam (630–660 $m\mu$), 0.254 microeinsteins/min., equivalent actinometrically to 5.7 mm.^{3} O_2/min.

No. I. 0.2 cc. *N* NaOH in each side-arm, gas phase air.

(Vessel volume 18.87 cc., liquid volume 7.40 cc., k_{O_2} 1.09)

20′ dark	−40 mm.	x_{O_2} = +43.5 mm.^{3}
20′ red light	−39 mm.	
20′ red light action	−1 mm.	x_{O_2} = +1.09 mm.^{3}

$$\frac{h\nu}{O_2} = \frac{20 \times 5.7}{1.09} = 105$$

No. II. Gas phase 5% CO_2 in air, respiration not compensated by white light.

	Vessel 3	Vessel 5	
Total volume	17.99	13.91	
Liquid volume	7.00	7.00	
k_{O_2}	1.046	0.665	
k_{CO_2}	1.634	1.253	
20′ dark	−13 mm.	−27.5 mm.	x_{O_2} = −40.6 mm.^{3}
20′ red light	−2 mm.	−7.0 mm.	
20′ red light action	+11 mm.	+20.5 mm.	$\left\{\begin{matrix} x_{O_2} = +23.8 \text{ mm.}^3 \\ x_{CO_2} = -19 \text{ mm.}^3 \end{matrix}\right\} \gamma = \frac{CO_2}{O_2} = -0.8$

$$\frac{h\nu}{O_2} = \frac{20 \times 5.7}{23.8} = 4.8$$

No. III. Gas phase 5% CO_2 in air, respiration overcompensated several-fold by white light.

	Vessel 3	Vessel 5	
20′ white light	+39 mm.	+57.5 mm.	
20′ white + red light	+48 mm.	+76 mm.	
20′ red light action	+ 9 mm.	+18.5 mm.	$\left\{\begin{matrix} x_{O_2} = -26 \text{ mm.}^3 \\ x_{CO_2} = -26 \text{ mm.}^3 \end{matrix}\right\} \gamma = \frac{CO_2}{O_2} = -1.0$

$$\frac{h\nu}{O_2} = \frac{20 \times 5.7}{26} = 4.4$$

It will be observed that the oxygen consumption in the dark in Nos. I and II is essentially the same (about 42 mm.³/20′). This means that the CO_2 pressures used did not influence respiration, and that the CO_2 pressure required to obtain maximum respiration is below that required for effective photosynthesis, where CO_2 is required as substrate and not merely catalytically.

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Myosmine in Cigar Tobacco

In a previous publication (1), an analytical procedure was described, which permits the separation of the alkaloids present in tobacco leaves from the alkaloid transformation products (1,2) which accumulate during the fermentation of the leaves. Fraction A of this analytical procedure, obtained by extraction of powdered tobacco in presence of MgO with a suitable hydrocarbon solvent, contains exclusively the alkaloids proper, free of any degradation products.

Detailed analyses of Fractions A of the same and different samples obtained from unfermented tobacco leaves have proved the presence of considerable amounts of nicotine and smaller amounts (7–10% of the nicotine) of nornicotine.[1] Invariably, the sum of these two alkaloids is slightly lower than the total alkaloids determined in separate subfractions. This indicates that other alkaloids are present in amounts of roughly 0.5–2% of the nicotine.[1] Various qualitative observations made it appear likely that these additional alkaloids are dehydrogenation products of nicotine and of nornicotine containing double bonds in their pyrrolidine rings. Since dehydrogenation products may represent intermediates between the alkaloids proper and their oxidation products which have been found in fermented leaves, attempts were made to identify these compounds.

According to Haines, Eisner and Woodward (3), myosmine (2-(3-pyridyl)-Δ^2-pyrroline) (4), in aqueous solutions, yields the correct

[1] These percentages, based on nicotine, are valid for unfermented tobacco only. The variable decrease of nicotine on fermentation prevents our expressing a similar ratio for fermented leaves.

value for the one primary amino group of its hydrolyzed form (with open pyrroline ring) by the van Slyke procedure. Model experiments proved[2] that this reaction can be employed with good results for the quantitative determination of amounts as low as 200 γ of myosmine (containing 20 γ of amino nitrogen), in the presence of very much larger amounts of nicotine, nornicotine and other alkaloids. Tested by this method, the following values were obtained for the myosmine content of various tobacco samples.

TABLE I

Alkaloid Contents of Various Tobacco Samples Expressed as Alkaloid Nitrogen[a] in Per Cent of Dry Weights of the Samples

No.	Type of tobacco sample	Nicotine nitrogen	Nornicotine nitrogen	Myosmine nitrogen by van Slyke method	Total alkaloid nitrogen other than nicotine[c] and nornicotine[c]
1	Pa. Seedleaf #5 unfermented	0.701_0	0.046_5	$0.011_6 \pm .004_7$ [14][b]	0.007–0.022
2	Pa. Seedleaf #5 fermented	0.310_5	0.030_2	$0.012_9 \pm .006_0$ [10][b]	0.006–0.020
3	Pa. Seedleaf #12 unfermented	0.712	0.050	$0.005_2 \pm .000_6$ [2][b]	— —
4	Pa. Seedleaf #12 fermented	0.246	0.024	$0.007_3 \pm .000_5$ [2][b]	— —
5	Pa. Seedleaf #24 unfermented	0.796	0.047	$0.015_0 \pm .003_0$ [3][b]	— —

[a] The values in terms of alkaloid nitrogen rather than of alkaloids permit an immediate nitrogen balance. The alkaloid values can be obtained by multiplying the values listed by the factor 5.8 for nicotine, 5.3 for nornicotine, and 5.2 for myosmine.

[b] The figures in brackets indicate the numbers of independent analyses made for each sample.

[c] Being a small difference between two large analytical values, this kind of determination is subject to a wide margin of error.

Some further samples of cigar leaf tobacco types, other than Pennsylvania Seedleaf, yielded similar values for myosmine nitrogen. Contrary to nicotine and nornicotine, the myosmine does not seem to decrease during fermentation. This may indicate its function as an intermediate. In spite of the fairly good reproducibility of these analytical

[2] Many of the pure alkaloids used for these model experiments were kindly supplied by Drs. Woodward, Eisner and Haines of the Eastern Regional Research Laboratory, Philadelphia 18, Pa.

results, a fully satisfactory identification of myosmine still has to be achieved.[3] Thus far, this compound was only identified in tobacco smoke (5) and as a product of the high temperature catalytic decomposition of nicotine (6).

Ultraviolet absorption spectra measured for various samples of Fraction A show deviation from the spectra (7) calculated for their nicotine and nornicotine contents. The shift of the spectra caused by these deviations may be interpreted as being caused by the presence of small amounts of myosmine and possibly further unsaturated alkaloids, but the presence of additional ultraviolet absorbing substances prevents a reliable quantitative evaluation.

Hydrogenation of the substances contained in Fraction A has yielded, in some tentative experiments, small increases of distillable nicotine. This effect would indicate the presence of N-methylmyosmine (8) which, on hydrogenation, yields nicotine. Myosmine, if hydrogenated, yields nornicotine which does not distil under the conditions employed in our analysis. Metanicotine would yield a similar hydrogenation effect as would myosmine. The order of magnitude of these additional compounds appears to be similar to that of myosmine.

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[3] Such an identification appears desirable because other unsaturated alkaloids related to myosmine may also respond to the van Slyke reaction.

Book Reviews

Nature of Life: A Study on Muscle. By A. SZENT-GYÖRGYI, Department of Biochemistry, The University, Budapest. Academic Press Inc., New York, N. Y. 1948. 91 pp. and 7 plates. Price $3.00.

Prof. Szent-Györgyi has that rare gift, which we remember in Gowland Hopkins, of being able to make a singular contribution to any field of biochemistry in which he choses to work. It is not merely that he makes a noteworthy contribution: many have that gift. But few have the talent to produce views and facts which demand a complete reorganization of a field. In his latest book Szent-Györgyi demands a complete reorganization of our views on the biophysics and biochemistry of muscular contraction. It is the subtitle and not the title which describes the contents. On the nature of life he has little to say except that since "life is characterised by self-reproduction, one rabbit could not be alive at all—only two rabbits are one rabbit." One day perhaps the author will write us another book which will be two rabbits. But, for the moment, a book which is one rabbit will do very well.

Muscle differs from most other cell types in that it has been the object of much detailed study at many different levels of organization. Emphasis is laid upon the distinctive attributes of these different levels, and upon the desirability of being alert to the emergence of properties at the higher levels of organization which would not readily be predicted from knowledge of the lower levels.

Falling into this category is Szent-Györgyi's suggestion that in a protein molecule there may be a tendency towards non-localization of electrons, which may occupy levels characteristic of the molecule as a whole. To the extent to which this is true there will be resonance energy, which is likely to be lost by changes in the configuration of the molecule. It is possible that in organized systems of proteins, electrons may even occupy levels which are characteristic of the organization. It may be that the characteristic properties of muscle are bound up with the properties of such systems.

No attempt is made to account for all the cytological characteristics of muscle fibrillae. The feature which is presented is of a structure based on two proteins, actin and myosin. Actin forms long threads composed of strings of globular molecules: myosin molecules, which are rigid rods, adsorb upon the actin threads to give actomyosin. A gel of actomyosin, when brought into contact with adenosine triphosphate (ATP), contracts vigorously and reversibly. The active system contains ATPase, and when the ATP is destroyed the contraction may reverse. The association of contractility with ATP is one of the main factors which leads Szent-Györgyi to postulate that the contractile unit in muscle is actomyosin. The myosin unit is itself a protein complex, for it appears to consist of a protein rod, which might perhaps be called structural myosin, onto which is adsorbed a variety of globular proteins, including ATPase, ADPase, ATP deaminase, *etc.* Without at least some of these adsorbed proteins, structural myosin is unable to give an active (contractile) complex with actin.

The picture which is presented is, on the biochemical level, uncommonly plausible. But cell physiologists will not be as contented with the picture as biochemists. The explanation of rigor which is suggested does not carry conviction, and there is one basic assumption in the whole story for which there is hardly a shred of evidence: this is that the system actomyosin–ATP is, in fact, involved in muscle contraction in the same way as it is with the contraction of an actomyosin gel in a test-tube. For example there is no evidence that ATP is, in fact, involved in the actual contraction and relaxation of muscle, rather than with recovery. That this is so makes a reasonable working hypothesis: but Szent-Györgyi, in common with many other biochemists, is inclined to treat it as a fact. As A. V. Hill has recently emphasised, this will not do.

But, as was said at the beginning of this review, this is a really valuable book, one which merits much thought. We can do with more such books.

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Textbook of Endocrinology. By HANS SELYE, Professor and Director of the Institut de Médecine et de Chirurgie experimentales, Université de Montréal, Montreal, Canada. Acta Endocrinologica Université de Montréal, Montreal, Canada. xxxii + 914 pp. Price $12.80.

Selye has achieved his main goal of providing a textbook of endocrinology "primarily for the medical student and physician." Others, even specialists, should find this book useful as a ready source of reference on the various aspects of endocrinology. The cost of the book, however, may prohibit its purchase by medical students, particularly since only a very short period of time is allotted to the teaching of endocrinology by most medical schools. On the other hand the general usefulness of the book for both experimental and clinical information should prompt its purchase by many.

The amount of space devoted to each subject seems to be judiciously chosen. The topics are well organized and clearly presented. Much of the material has been submitted to colleagues and associates for criticism and revision.

In the Introduction, Selye discusses the tasks undertaken by his institute "in an attempt to delimit and systematize the field of endocrinology" and the role of this book in this general program.

The Introduction is followed by a section entitled General Endocrinology. In 37 pages, the author presents a brief and concise description of the general nature and action of the endocrine organs, the classical and recognized methods of study, a brief history of endocrinology prior to the twentieth century, a list of journals and monographs containing endocrine literature and a compilation of the commercially available endocrine preparations with the names of the respective manufacturers.

Chapter I (43 pages) is devoted to a discussion of the occurrence and role of steroids, their chemistry, chemical structure (well illustrated by spatial models), nomenclature, chemical structure as related to biological activity, biogenesis and metabolism of the steroid hormones, including a valuable tabulation of steroids isolated from natural sources.

In the next nine chapters the endocrine organs are treated in the following sequence: Adrenals (109 pages). Unfortunately, Selye has chosen to treat the cortex and medulla simultaneously. Thus, continuity and clarity have suffered. The same criticism may

be made of the Pituitary (121 pages), the various lobes of which should have been treated separately; Ovary (155 pages); Pancreas (58 pages); Parathyroid (54 pages); Pineal (6 pages) Testes (76 pages); Thymus (10 pages); and Thyroid (86 pages). The general plan of treatment is the same for each chapter. The various subjects are considered in a logical sequence: Historic Introduction; Normal Morphology (anatomy, histology, comparative morphology, embryology, and theories concerning the histophysiology of the organ); Chemistry of the Organ; General Pharmacology of the Hormones (standardization, pharmacology, mode of administration, and chief indications); Experimental Physiology (explantation, transplantation, technic of extirpation, effects of extirpation, and treatment with hormones); Metabolism of the Hormones including content in the body fluid and tissues in health and disease; Stimuli Influencing Structure and Activity of the Organ; Diseases of the Organ; Hypo- and Hyper-Activity and Tumors. Approximately 50% of the space is allotted to clinical material.

The various gastrointestinal hormones, other postulated hormones, and the hormones of invertebrates and plants are presented in a brief but adequate manner in the 19 pages of Chapter 10.

In the eleventh and last chapter interrelationships among various hormones are discussed with excellent schematic diagrams. Most of the space is allotted to the female reproductive system, pregnancy and lactation (39 pages), and the adaptation syndrome (30 pages). The last three pages contains a General Survey of Hormonal Correlations with an excellent but somewhat overburdened diagrammatic sketch. This portion is worthy of more consideration.

The book is profusely illustrated with not only clinical abnormalities but also photographs and graphs to illustrate many physiological and pathological effects of the hormones. Diagrammatic schemes are used frequently to clarify or correlate specific effects. The author has avoided the cataloging of a large number of references but instead has chosen to provide at the end of each chapter a number of key references in the form of reviews, monographs and occasionally an original article. An extensive index is included.

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Physiology of Man in the Desert. By E. F. Adolph and Associates. Interscience Publishers, Inc., New York, N. Y., 1947. xiii + 357 pp. Price $6.50.

The book is a real contribution in a field of physiology in need of information, a field in which commonly accepted beliefs are at such variance with the facts that they are a hazard to all but experienced desert travelers. "Contrary to the popular legend of the wanderer tortured by killing thirst, when the unfortunate victim of water shortage is first incapacitated, he is neither delirious nor in agony from 'mouth thirst'; he is simply incapable of even mild physical effort."

The book also laid to rest the popular beliefs that in deserts men (1) drink more water than they need, (2) can be trained to do with less water, and (3) that men should drink no water at work or on the march. So firmly established has been the notion that men drink more water than they need that a plan to ration desert troops to 2 quarts of water daily was abandoned only when the commanding general was induced to try it. The decision was that shortage of water was not consistent with field operations.

Man may acclimatize to heat, but he cannot adapt himself to less water. On the contrary, the real danger is that, as man dehydrates, he does not voluntarily drink enough water to replace that lost, and men have succumbed to dehydration exhaustion with plenty of water in their canteens.

The writers have given a detailed picture of the signs and symptoms of dehydration. "Rarely is lack of body water recognized as the major cause of reduced efficiency in the desert; the blame is often placed on the heat, or diet, or the difficulty of the task set—impaired morale is one of the earliest signs of dehydration—when fully hydrated, man cheerfully does tasks which he finds distasteful if he is moderately dehydrated and consequently in low spirits."

The book evaluates the 3 stresses which the desert dweller must contend with, namely, work, heat, and dehydration. The fully hydrated man can turn out 77% of the work he is capable of at moderate temperatures, at 110°F., but only 37% of the work when dehydrated by the loss of water equal to 4.0% of his body weight.

There are many fine physiological contributions in the book involving cardiac output and changes in the composition of many tissues with progressive dehydration. Of special interest are two physiological processes, heat regulation and urine production, for which body water is needed. Water is available for sweat formation (heat regulation) and urine production when the body is severely dehydrated. The body temperature and body composition must remain fairly constant, regardless of other possible physiological derangements caused by the loss of water. While most of the information was obtained on human subjects, animals were also used in their investigations.

Thirst received considerable study and interesting species differences came to light. Thirst served as an accurate indicator of the water needs of the dog and he replaced the water he lost by drinking an almost equivalent amount. In man, thirst seemed to be inhibited when dehydrated more than 2.0%, so that, when most needed, thirst fails him as an indicator of his water requirements. Before man voluntarily makes good his water losses, food and leisure seem to be essential.

Maps are drawn which show average amounts of water required for the maintenance of one man in the hottest month in the desert areas of the United States, Northern Africa, Asia, and Australia. There are also other useful maps and tables. One table shows the mileage per gallon of water that the desert traveler is capable of under various conditions.

It is unfortunate that a book containing so much valuable information should be so poorly put together. Actually it is not a book but a collection of about 20 independent contributions. The result is that the book is badly repetitious. It could have been integrated into a book of half its present size with great improvement. Many chapters are excellent, some are fair and at least one, Chapter 7, is almost entirely excess baggage.

The book as a whole supplies much badly needed information and is a most welcome addition to our knowledge on the physiology of man in the Desert.

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Vitamins and Hormones, Vol. VI. Edited by ROBERT S. HARRIS, Massachusetts Institute of Technology, and KENNETH V. THIMAN, Harvard University, Cambridge, Massachusetts. Academic Press, Inc., New York, N. Y., 1948. xi + 435 pp. Price $7.80.

Vol. VI of *Vitamins and Hormones* contains 8 reviews on current topics and a useful cumulative index for Vol. I–V. The large number of reviews which have recently appeared in this general field make it necessary for some of the reviewers to confine their efforts to specific periods of time or limited phases of the subject matter.

The review of the chemistry and biological action of pteroylglutamic acid and related compounds by Brian L. Hutchings and John H. Mowat aids one in unraveling this complicated subject. It is of somewhat greater usefulness in clarifying the chemistry than the biological action of these compounds.

The review of Vitamin K by Henrik Dam serves as a valuable addendum to previous works on this subject. B. S. Schweigert has gathered together valuable practical information for those interested in the use of the cotton rat and hamster in nutritional experimentation.

The difficulties of definition of vitamins as pharmacological agents are pointed out by Hans Molitor and Gladys A. Emerson. The authors succeed in bringing together a large number of relatively unrelated observations in an interesting manner. The common practice of using relatively large doses of vitamins makes a knowledge of the pharmacological properties of these compounds of some importance.

H. M. Sinclair discusses in some detail the problems of assessing nutritional status of the human in the absence of manifest clinical signs and symptoms. It is obvious from his discussion that much remains to be done before simple criteria for such studies are developed. Van Lanen and Turner have reviewed in some detail the occurrence and synthesis of vitamins in microorganisms. The subject is reviewed primarily from a descriptive rather than analytical point of view.

The review on B vitamins as plant hormones by James and Harriet Bonner is an excellent summary of work in this field. The correlation of activities of various compounds in plants, microorganisms and mammals is of great inetrest from the point of view of comparative biochemistry.

The extensive review by Edward C. Kendall of the role of the adrenal cortex on the metabolism of water and electrolytes is perhaps the most useful of the group. The great increase in interest in adrenal physiology in recent months should make this summary particularly valuable.

ALBERT DORFMAN, Chicago, Ill.

Studies on the Action of Chloramphenicol (Chloromycetin[1]) on Enzymatic Systems. I. Effect of Chloramphenicol on the Activity of Proteolytic Enzymes

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Introduction

With the isolation and identification of the new antibiotic chloramphenicol (6,10,25) from culture filtrates of a *Streptomyces*, it seemed desirable to investigate the effects of this drug on isolated and intact enzymatic systems to determine, if possible, the mode of action of this new compound.

The establishment of the structure of chloramphenicol by Rebstock *et al.* (9, 17,23) as D(−)threo-1-*p*-nitrophenyl-2-dichloracetamido-1,3 propanediol suggested the possibility that the compound might be acting as an antimetabolite, replacing the normal peptides which were being utilized by the bacteria which are sensitive to this antibiotic. In this case, the compound would probably act as an enzymatic inhibitor for the proteolytic enzymes. It was therefore decided to test the inhibitory action of chloramphenicol on proteolytic systems. The present paper deals with some of the observations that were made on this subject.

Since the proteolytic enzymes which are known to occur in bacteria differ from the corresponding enzymes of animals and higher plants, as has been pointed out by various investigators (12,22,26), it seemed desirable to study the effects of chloramphenicol on those enzymes which can be obtained in crystalline form from animals and higher plants, as well as on the enzymes isolated from bacteria which are sensitive to the antibiotic. The former group of enzymes would give a more direct insight into the action of the antibiotic, since a considerable amount of data is available as to the chemical nature of the peptide molecules which are hydrolyzed by these enzymes. The data obtained

[1] Parke, Davis & Co. trade mark.

from these studies would also indicate whether the differences in the response exhibited by animal cells and bacterial cells to chloramphenicol can be explained on the basis of the effects of the antibiotic on their respective proteolytic enzyme systems.

Experimental

The effects of chloramphenicol on isolated proteolytic enzymes from plants and animals were investigated by using crystalline trypsin (20), chymotrypsin (18), pepsin (21), and papain (5). The changes in enzymatic activity were followed by using methods recommended by Anson *et al.* (2,3,4). Casein was used as the substrate in the case of trypsin, chymotrypsin, and papain, while hemoglobin was used as the substrate for pepsin.

The effects of chloramphenicol on each of these enzymes were determined by estimating the decrease or increase in the activity of a standard enzyme solution after the treatment with the antibiotic. One ml. of the enzyme solution was mixed with 1 ml. of the chloramphenicol solution containing from 3.2 to 161.5 γ of the antibiotic/ml. The solution was allowed to stand for 30 min. at 37°C. to allow sufficient time for the antibiotic and enzyme to react before determining the activity.

The typical effects of chloramphenicol on crystalline trypsin, chymotrypsin, pepsin and papain are shown in Table I. The enzymatic activity has been expressed in these cases in terms of the mg. of tyrosine liberated from the substrates by enzymatic digestion. The results were identical with all concentrations of chloramphenicol tested.

The proteolytic enzymes produced by bacteria are liberated into the culture medium and are generally isolated from the medium during the commercial production of

TABLE I

The Effect of Chloramphenicol on Crystalline Proteolytic Enzymes

Enzyme	Concentration of chloramphenicol	Tyrosine liberated
	γ/ml.	*mg.*
Trypsin	0	0.41
Trypsin	6.4	0.41
Trypsin	12.8	0.41
Trypsin	161.5	0.42
Chymotrypsin	0	0.57
Chymotrypsin	6.4	0.58
Chymotrypsin	12.8	0.58
Chymotrypsin	161.5	0.57
Pepsin	0	0.39
Pepsin	6.4	0.39
Pepsin	12.8	0.39
Pepsin	161.5	0.39
Papain	0	0.20
Papain	6.4	0.21
Papain	12.8	0.21
Papain	161.5	0.20

bacterial proteases (26). At the same time, there are proteolytic enzymes which remain within the bacterial cell and carry on the normal metabolic processes (8). From the data that are now available, it is impossible to ascertain whether the proteolytic enzymes liberated into the culture medium are identical with those enzymes remaining within the cell. Therefore, the effects of chloramphenicol were studied on proteolytic enzymes isolated from the culture medium and also the enzymes obtained from the bacterial cells by autolysis or drying and defatting with acetone.

The test organisms, *B. mycoides, B. subtilis, and E. coli*, were grown in beef infusion broth for 6 days at 37°C. until a luxurious growth was obtained. The bacterial cells were then removed from the liquid phase by passing the culture solution through a Seitz filter. The proteolytic enzymes which have been liberated into the medium passed through the filter and were collected in the filtrate. The effect of chloramphenicol on these extracellular proteases was then tested by incubating 10 ml. of the filtrate with 1 ml. of chloramphenicol solution and 100 ml. of 3% gelatin solution for 72 hr. at 37°C. under sterile conditions. The extent of hydrolysis of the gelatin was then determined by using the formalin titration procedure of Søensen.

TABLE II

The Effect of Chloramphenicol on Proteases Produced by B. subtilis

Enzyme	Concentration of chloramphenicol γ/*ml.*	0.1 *N* NaOH *ml.*
Extracellular protease	100.0	12.10
Extracellular protease	25.0	12.07
Extracellular protease	3.1	12.08
Extracellular protease	0.4	12.08
Extracellular protease	0.0	12.10
Cell autolyzates	100.0	3.70
Cell autolyzates	25.0	3.65
Cell authlyzates	3.1	3.65
Cell autolyzates	0.4	3.60
Cell autolyzates	0.0	3.65
Acetonized cells	100.0	6.20
Acetonized cells	25.0	6.20
Acetonized cells	3.1	6.20
Acetonized cells	0.4	6.25
Acetonized cells	0.0	6.25

The data obtained from a typical experiment using filtrates of *B. subtilis* cultures is given in Table II. The extent of hydrolysis of the gelatin is expressed in terms of ml. of 0.1 *N* NaOH solution. Similar results were obtained with concentrated enzyme solutions prepared from the filtrates by methyl alcohol fractionation.

A solution of bacterial proteases was prepared from the bacterial cells by liberating the proteases from the cells by autolysis. The cells collected from the cultures were washed 4 times with physiological

saline solution and suspended in distilled water containing toluene. The suspension was shaken for 1 hr. and then allowed to stand at room temperature for 5–7 days. The suspension was centrifuged and the clear supernatant used as the enzyme solution.

The effect of chloramphenicol on these proteases was tested by using a mixture of 1 ml. of the autolyzate, 1 ml. of the chloramphenicol solution and 50 ml. of the gelatin solution according to the procedure previously described. The mixture was incubated 16 hr. and the extent of hydrolysis then determined by the formalin titration procedure. An example of the results obtained are presented in Table II. In this case the enzyme preparation was prepared from *B. subtilis* cells.

Acetonized cell preparations of the test organisms were prepared by suspending the washed cells in glass-distilled acetone for 6 hr. at 3°C. The acetone was changed every 2 hr. and replaced with another portion of cold acetone. The cells were then suspended in a mixture of 50% acetone and 50% ethyl ether for 1 hr., and finally in ethyl ether for 1 hr. The cells were then dried at room temperature in a hood until all traces of acetone and ether had been removed. The material was then ground in a glass mortar using a small quantity of powdered glass. The material was suspended in distilled water and used as the enzyme solution. The effect of chloramphenicol on this preparation using the procedure outlined above is shown in Table II.

Discussion

In the experiments which have been reported above, concentrations of chloramphenicol have been employed which would approximate bacteriostatic and subbacteriostatic concentrations of the drug if used with organisms possessing the same concentration of enzyme used in these studies.

In the initial experiments with chloramphenicol, the plate method suggested by Gorini *et al.* (13) was used to determine the inhibitory action of the antibiotic on the proteases liberated by the test organisms into the culture medium. Stone's agar was used in preparing the assay plates. In these experiments, the same results were obtained as reported by Gorini *et al.* (13) when they tested penicillin. The zones of inhibition appeared very turbid indicating that the gelatin had not been hydrolyzed by the bacteria present in these zones and, therefore, chloramphenicol had decreased the proteolytic activity in these areas. The enhanced growth ring appeared as a transparent halo around the zone

of inhibition. Since the gelatin has been completely hydrolyzed in this area, this would seem to be the zone of most intense proteolytic activity. The background area appeared slightly turbid suggesting partial hydrolysis of the proteins present. These results reported above would at first seem to indicate that chloramphenicol inhibits the proteolytic activity of the enzyme liberated into the medium by bacteria. If, however, the number of bacteria present in each zone are taken into consideration, it would appear more likely that the differences observed were due to the difference in the number of bacteria present in each region. Since there are no indications in this method that cessation of growth was due to inhibition of proteolytic activity, and since the quantity of proteases produced by the small number of bacteria in the zone of inhibition are not sufficient to be detected by this method, it is very doubtful that the method shows that chloramphenicol is inhibiting bacterial proteases.

From the results obtained from the studies on the effect of chloramphenicol on proteolytic enzymes, it is apparant that although chloramphenicol is related to peptides which are split by proteolytic enzymes (7) the amide group of this compound is not able to block the reaction between these enzymes and their normal substrates. In fact the chloramphenicol itself appears to be attacked by these proteolytic enzymes (24). It has been found that papain and trypsin will hydrolyze the amide linkage of chloramphenicol to a slight extent while bacterial proteolytic enzymes are very effective in these regards.

From the investigation of Herriott *et al.* (14,15,16) it has been found that the phenolic hydroxyl groups and benzenoid portion of the tyrosine molecules present in pepsin are important for enzymatic activity. It appears, therefore, that chloramphenicol is not blocking these groups or modifying the structure of the benzenoid portion of the molecule by transferring chlorine atoms to it.

The results obtained with papain also indicated that the antibiotic is not influencing the relationship between the disulfide and sulfhydryl groups of the enzyme molecule which are important for its action (11,19).

Summary

1. The inhibitory effects of the new antibiotic chloramphenicol have been tested on a series of proteolytic enzymes to determine whether the effects of this compound on gram negative bacteria can be explained in

terms of effects of the drug on proteolytic enzymes present in the bacteria.

2. Data have been presented to indicate that chloramphenicol does not inhibit or activate bacterial proteases, crystalline trypsin, chymotrypsin, pepsin, or papain when used in subbacteriostatic and bacteriostatic concentrations.

3. The results indicated that chloramphenicol does not block the phenolic hydroxyl groups, amine groups, carboxyl groups, or the $-SH$ groups in proteolytic enzymes tested, and which are necessary for enzymatic activity.

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A Microanalytical Method for the Volatile Fatty Acids [1,2]

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Introduction

The usual methods for estimating the volatile fatty acids involve separation of these substances from the sample by steam distillation and titration with standard alkali (1). In the method to be described, distillation is replaced by a microdiffusion procedure with several resultant advantages. Large numbers of samples can be analyzed simultaneously with simple apparatus and a minimum of manipulation. The acids are collected in a very small volume in which an accurate microtitration can be made.

Apparatus

Microdiffusion Unit

The microdiffusion vessel is a 50 ml. glass-stoppered Erlenmeyer flask (Fig. 1). The steel center cup is spun from 0.01 in. stainless steel (No. 302).[3] Its inner surface is well polished. It is $\frac{3}{16}$ in. deep and has an outside diameter of $\frac{1}{2}$ in. The radius of curvature between the wall and floor of the cup is $\frac{1}{16}$ in. These cups are cleaned by boiling 10–15 min. in dilute (1–3%) acetic acid, rinsing copiously in water, and drying in an oven. The steel cup is held in one of glass made by cutting the lower 15 mm. portion from a shell vial (15 mm. O. D.).

Microburette

All liquid quantities of 0.1 ml. or less are delivered with a microburette constructed from a tuberculin syringe and a micrometer caliper (Figs. 2 and 3). This instrument

[1] This work was aided in part by a grant to Professor E. S. Guzman Barron by the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

[2] A portion of this work was described before the meeting of the American Society of Biological Chemists at Atlantic City, N. J., March 16, 1948.

[3] These cups were obtained from Mr. Lee A. Farmer of the M and L Instrument Co., 1307 E. 71 Place, Chicago 19, Ill.

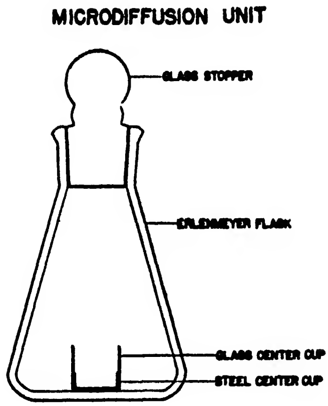

Fig. 1.

embodies principles used by Dean and Fetcher (2). To make the burette, a $\frac{1}{2}$ in. section of the needle end of the syringe barrel is slowly warmed (2–3 min.) to red heat, then drawn out about 4 in. with the aid of a glass rod. A right angle bend is made near the origin of the drawn portion and the syringe placed in an upright position in a muffle furnace previously brought to 600°C. After 20–30 min. the current is shut off and the furnace allowed to cool slowly to room temperature. At a point about 3 in. from the bend, the drawn glass is heated in a small flame over a length of about $\frac{1}{4}$ in. and drawn out until it is sealed. It is then cut at a point just above the seal. If the orifice is too small the tip is ground on an abrasive stone until it is adequate. With

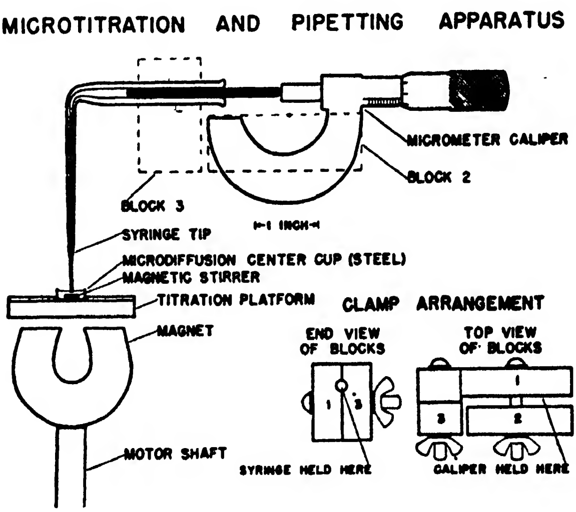

Fig. 2.

an opening of proper size it is possible to fill the syringe with 0.5 ml. of water in 10–15 sec. by pulling the plunger gently.

To calibrate the burette the diameter of the plunger is measured at its widest point and the cross-section area calculated. This value in mm.^{2} is equal to the number of mm.^{3} delivered when the plunger is advanced 1 mm. The delivery error with 1 ml. syringes is about 0.1 mm.^{3}

The most inexpensive micrometer is adequate for constructing this burette. A small portion of the jaw of the caliper, including the anvil, is cut away and the syringe attached with the aid of a clamp made from 3 wooden blocks, as illustrated in Figs. 2 and 3.

Titration Assembly

The microburette is held in a ringstand clamp as shown in Fig. 3. The shaft of this clamp is horizontal; it is perpendicular to the syringe barrel and is held by a second clamp in such a way that is can rotate when the tip of the syringe is raised or lowered.

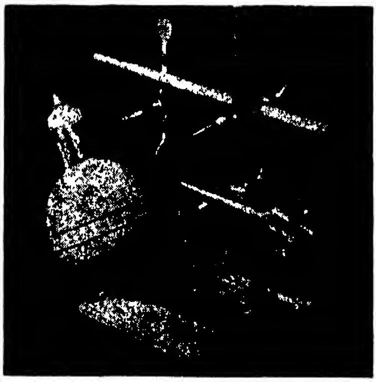

FIG. 3.

The titration platform consists of two small boards nailed together. The upper one is about $\frac{1}{16}$ in. thick and has a hole slightly larger than $\frac{3}{8}$ in. in diam. drilled through its center. This serves as a pit for the steel cup and prevents it from spinning or migrating about the platform during a titration. When glass cups are used, this platform is unnecessary. An inverted spot plate, which provides a white background, is more suitable.

An Alnico magnet (40 mm. long, 46 mm. wide) is attached to the shaft of a small variable speed motor (not shown in Figs.). This motor and magnet are mounted on a separate ringstand so that vibrations are not communicated to the rest of the apparatus. The position of the magnet is shown in Fig. 2.

The interior of the steel cup must be brightly lighted during a titration. The lamp (Fig. 3) must be turned off between titrations to prevent warming the solution in the burette.

The magnetic stirrer is made by imbedding a small piece of iron wire ("stove-pipe" wire) in glass.

Operation of Burette

Cups into which reagents are to be measured are held in a Petri dish on the titration platform. To deliver a specified volume of solution the burette tip is brought to rest on the cup floor and the micrometer shaft advanced the number of mm. required to give the desired volume. The tip is then immediately raised to avoid the possibility of additional fluid flowing out due to capillarity.[4] During a titration the individual cup is held on the platform as shown in Figs. 2 and 3. With the light and stirring motor switched on and a magnetic stirrer in the cup, the burette tip is placed just below the surface of the liquid. Solution is then run in from the burette until the end point is observed against the bright steel surface. The syringe is then immediately raised. The volume of solution consumed in the titration is calculated by multiplying the cross section area of the syringe plunger by the number of mm. it advances, *e.g.*, the initial minus the final micrometer reading.

Reagents

For Basic Procedure

0.03 N $K_2CO_3 \cdot 1.5H_2O$. Dissolve 0.124 g. of $K_2CO_3 \cdot 1.5\ H_2O$ (C. P. Baker's) plus 0.25 g. of KNO_3 in distilled water, and dilute to 50 ml.

80% H_2SO_4. Add 400 ml. of conc. H_2SO_4 (C. P. or Reagent) to 100 ml. of 0.0005 *M* acetic acid. This solution must be well mixed. When steel center cups are not used, 100 ml. of distilled water may be substituted for the 0.0005 *M* acetic acid.

0.1 M Monosodium Maleate. Dilute 5 ml. of 1.0 *N* NaOH and 1.5 ml. of isoamyl alcohol plus 0.58 g. of maleic acid (C. P. Pfanstiehl) with distilled water to 50 ml.

0.1 N Standard NaOH.

Indicator Solution. One ml. of phenolphthalein indicator, 0.1% in 95% alcohol, is diluted to 25 ml. with CO_2-free (boiled) distilled water. This solution should be made up on the day of use.

Ag_2SO_4 (C. P. or Reagent).

For Ether Extraction Procedure

5 N H_2SO_4.

Alkaline Na_2SO_4. Twenty g. of anhydrous Na_2SO_4 (C. P. or Reagent) plus 10 ml. of 1 *N* NaOH are dissolved and diluted to 100 ml.

Ethyl Ether. This reagent should be of good quality, substantially free of peroxides.

For Oxidative Procedure

Alkaline Lactate. Dissolve 0.4 ml. of Reagent lactic acid in 100 ml. of 1.0 *N* NaOH.

Saturated $KMnO_4$. Dissolve 7 g. of $KMnO_4$ (Reagent) in hot distilled water. Allow to cool to room temperature and use supernatant solution.

0.1 M $SnSO_4$. Dissolve 2.15 g. $SnSO_4$ (C. P.) in distilled water and dilute to 100 ml. This substance forms a fine precipitate immediately on dissolving. It settles very slowly, however, and can be pipetted and used as a suspension.

[4] A rubber band properly attached to the plunger eliminates this danger.

Experimental

Basic Procedure

The principle of the method is to separate the volatile acid by diffusion from the acidified sample in the main chamber of the microdiffusion unit to the alkaline solution in the center cup, then to estimate it in the cup by an appropriate microtitration. The procedure described below is suitable for acetic, propionic, and butyric acids. The details are exactly the same for the higher acids, such as caproic, except that the steel cup is omitted and the $K_2CO_3 \cdot 1.5H_2O$ solution placed directly in a glass cup (12 mm. O. D.).

Acids higher than butyric induce, during the diffusion process, a creeping of the alkaline solution over the surface and out of the steel cup, causing serious errors in some instances. Without the steel cup, errors occur which are due to interaction of the glass with the alkali, but these are less objectionable, rarely exceeding 0.07 microequivalent of acid. The glass cup must be discarded after one usage, for the rate of its reaction with $K_2CO_3 \cdot 1.5H_2O$ increases on repeated use. Cups of alkali-resistant glass (Corning No. 728) were, surprisingly, less satisfactory than those of the lime glass of which shell vials are made.

There is a tendency for small amounts of the alkali to creep out of the steel center cup in the blank run as well as in the presence of certain higher acids. Error from this source has been reduced to a negligible value by inclusion of KNO_3 in the alkaline solution, by inclusion of a trace of acetic acid in the H_2SO_4 added to the main chamber, and by rapid, rather than slow, heating of the unit in the oven.

The efficiency of the alkaline solution as a trapping agent is strongly affected by the solubility of the alkali used. KOH is rapidly converted to the carbonate when added to the center cup. The carbonate, K_2CO_3, is considerably less soluble than the hydrate, $K_2CO_3 \cdot 1.5\ H_2O$. The latter is the only agent which has permitted good recoveries of volatile acid over an extended period.

When the diffusion of volatile acid into the center cup is complete, a solution of monosodium maleate is used to neutralize the excess alkali in the cup and liberate CO_2. Isoamyl alcohol reduces the surface tension of the solution, allowing it to spread readily into a thin layer over the interior of the cup. While monosodium maleate is a sufficiently strong acid to liberate CO_2 and to be titrated to the phenolphthalein endpoint, it is too weak to liberate a substantial fraction of free fatty acids from their salts. The latter are, therefore, not lost by evaporation during the escape of CO_2.

In the steam distillation of fatty acids (1), salts such as $MgSO_4$ are used to increase the distillation rate. Salts were not found adequate, however, to promote rapid diffusion of these acids from the main chamber into the center cup of the apparatus described here. H_2SO_4, in relatively high concentration, was more satisfactory.

The details of the basic procedure are as follows: One ml. of an unknown solution, containing not more than 2 microequivalents of volatile acid, is pipetted into a glass-stoppered Erlenmeyer flask. One ml. of 80% H_2SO_4 is added and the flask shaken gently to ensure mixing. The glass-held steel center cup, containing 100 mm.[3] of $K_2CO_3 \cdot 1.5H_2O$

solution, is taken from a Petri dish and placed centrally on the floor of the flask with the aid of forceps (Fig. 1). The glass cup stands directly in the acidified sample. The glass stopper is put loosely in place (do not twist or "lock") and the flask set in a 100–105°C. oven. It should be set directly on the oven shelf or grating, not in a tray. After 14–16 hr. the center cup is removed and placed on a paper towel which absorbs the liquid from the under surface. The steel cup is then removed from the glass one by grasping from the inside with sharp-pointed forceps having the prongs bent outward, and placed in a Petri dish. Forty mm.^{3} of monosodium maleate solution are added to the cup and the dish tilted gently, if necessary, to ensure distribution of this reagent over the cup bottom. After 10–20 min., during which CO_2 escapes, about 0.3 ml. of indicator solution and a magnetic stirrer are added. The cup is placed on the titration platform and the titration performed as described above with 0.1 *N* NaOH.

TABLE I

Recovery of Individual Volatile Acids from Standard Solutions

Procedure described in text. Glass center cups were used for valeric, caprylic and caproic acids.

Microequivalents added	Microequivalents found					
	Acetic	Propionic	Butyric	Valeric	Caproic	Caprylic
0.10	0.09	0.10	0.09	0.13	0.07	0.15
1.00	0.98	1.00	1.03	1.03	1.00	0.87
2.00	2.03	2.05	1.97	1.97	1.92	1.65

The amount of volatile acid is calculated by subtracting a blank titration value from that obtained with the unknown. The theoretical blank is equal to the difference in the number of equivalents of maleate and $K_2CO_3 \cdot 1.5H_2O$ plus the trace amount of acetic acid present in 1 ml. of the 80% H_2SO_4.

Table I shows the recovery of 6 volatile acids from standard solutions by the basic procedure just described, and Table II shows the recovery of 3 acids from copper-lime filtrates of yeast.

If chloride is present in the sample, it will yield HCl to the center cup. This is prevented by addition of about 10 mg. of solid Ag_2SO_4 prior to the H_2SO_4 solution. The flask should be tilted and rotated several times to ensure distribution of the silver throughout the solution.

The basic procedure has been used with no significant interference from the following acids: acetoacetic, citric, isocitric, *cis*-aconitic, α-ketoglutaric, succinic, fumaric, and malic. Of 10 amino acids tested, none gave interference except tryptophan, which decomposed in the strong acid and yielded more than 0.5 microequivalent of volatile acid per micromole of the compound added. Lactic, pyruvic and oxaloacetic acids yielded 0.2–0.5 microequivalent of volatile acid per micromole, and β-hydroxybutyric acid 0.5–0.6 microequivalent. Glucose and carbohydrate-containing substances such as adenosine and its phosphates interfere seriously, to about the same extent per mole as β-hydroxybutyric acid. Solid $HgSO_4$, added to the flask, completely prevents diffusion of pyruvic acid.

TABLE II

Recovery of Volatile Acids from Copper-Lime Filtrates of Yeast

20 mg. dry wt. of washed bakers' yeast/ml. was suspended in a solution containing 0.02 M NaH_2PO_4 and 0.13 M NaCl. One ml. of this suspension was added to a centrifuge tube containing 7 ml. H_2O and one of the volatile acids (neutralized) in sufficient amount to give the indicated final ratio of acid to yeast. One ml. of 15% $CuSO_4 \cdot 5H_2O$ was added and mixed, then one ml. of a 10% $Ca(OH)_2$ suspension. After shaking and standing 15 min. the tubes were centrifuged. One ml. aliquots of the filtered supernatant solutions were analyzed by the basic procedure. About 10 mg. of Ag_2SO_4 were added to each microdiffusion unit prior to addition of the H_2SO_4. Blank: 0.12 microequivalent of volatile acid/2 mg. yeast.

Microequivalents added /2 mg. of yeast	Microequivalents found/2 mg. of yeast		
	Acetic	Butyric	Caproic
0.20	0.20	0.20	0.19
0.60	0.62	0.60	0.54
1.20	1.18	1.27	1.27

Ether Extraction

The volatile acids may be separated from the majority of interfering substances by extracting them from aqueous solution with ethyl ether. Table III shows the fraction of each of 6 acids extracted when 15 ml. of ether was used to extract 3.2 ml. of aqueous solution, as well as when the water and ether volumes were equal. It was found possible to quantitatively re-extract the acids from ether into a small volume of NaOH solution only when a large amount of Na_2SO_4 was added.

The precedure is as follows: Three ml. of a protein-free filtrate is placed in a 30 ml. glass-stoppered centrifuge tube.[5] Two-tenths ml. of 5 N H_2SO_4 is added, 15 ml. of ether run in, and the tube shaken vigorously for 2 min. Ten ml. of the supernatant ether is transferred with a pipette[6] to a second stoppered tube containing 1.2 ml. of the alkaline Na_2SO_4 solution. This tube is shaken for 1 min. One ml. of the aqueous layer is then transferred with a fine-tipped pipette to a 50 ml. Erlenmeyer flask and the analysis carried out as described above by the microdiffusion procedure.

TABLE III

Extraction of Volatile Acids from Water with Ethyl Ether

I. 15 ml. of ether used to extract 3.2 ml. of aqueous solution.[a] II. 10 ml. of ether used to extract 10 ml. of aqueous solution.

Acid extracted	Per cent of acid found in ether layer	
	I	II
Acetic	71	32
Propionic	87	60
Butyric	95	83
Valeric	98	93
Caproic	100	98
Caprylic	100	100

[a] The small amount of calcium in copper-lime filtrates causes additional retention in the aqueous layer of about 0.1 micromole of volatile acid; this amount is independent of the total volatile acid in the sample. Addition of 0.1 ml. of 10% Na_2WO_4 prior to extraction causes precipitation of the calcium (which need not be removed) and elimination of this source of error.

To calculate the amount of volatile acid present in the original 3 ml. of filtrate, the quantity found is divided by the factor 0.55 multiplied by whatever fraction of the acid in question Table III indicates should be extracted by this procedure. Table IV shows the recovery of 3 volatile acids from 3 different animal tissues by the procedure just described.

Carried through this procedure, lactic and pyruvic acids yielded 0.1–0.2 microequivalent of volatile acid, calculated as acetic acid, per micromole of either of these substances present in the filtrates. β-

[5] Maizel-Gerson reaction vessel. Obtained from the Wilkens-Anderson Co., 111 N. Canal St., Chicago, Ill.

[6] The tip of this pipette is bent 90° with respect to the pipette axis to avoid drawing up aqueous solution. One pipette is used for all transfers; a stream of air is drawn through it with a water aspirator after each transfer to clear it of ether and volatile acid.

Hydroxybutyric acid exhibits the same distribution ratio between water and ether as acetic acid, hence interferes to about the same extent as when filtrates are analyzed directly. If it is desired to use $HgSO_4$ or the $KMnO_4$ procedure (below), following the ether extraction, the alkaline Na_2SO_4 extract must first be evaporated to dryness in the flask, then 1 ml. of water added.

TABLE IV

Recovery of Individual Volatile Acids from Animal Tissues by Ether Extraction Procedure

The fresh tissue samples were homogenized in sufficient water to give a suspension with 0.15 g. tissue/ml. Two ml. of the suspension was added to a centrifuge tube containing 6 ml. ofwater and an amount of the volatile acid (neutralized) to give the indicated ratio of acid to tissue. The copper-lime precipitation was then carried out as indicated in Table II. Blank volatile acid values, calculated as acetic acid, were as follows in microequivalents/30 mg. fresh tissue: liver 0.11, brain 0.03, and muscle 0.07.

Microequivalents added 30 mg. of tissue	Microequivalents found/30 mg. of tissue								
	Rabbit liver			Rat brain			Rabbit muscle		
	Acetic	Butyric	Caproic	Acetic	Butyric	Caproic	Acetic	Butyric	Caproic
0.20	0.20	0.20	0.20	0.21	0.22	0.18	0.21	0.25	0.18
0.60	0.56	0.58	0.57	0.68	0.60	0.60	0.63	0.61	0.58
1.20	1.14	1.18	1.06	1.21	1.23	1.05	1.15	1.16	1.06

Analyses of Mixtures

Osburn, Wood and Werkman (3) have described procedures for analyses of mixtures of volatile acids in which the individual acids are distinguished on the basis of the differences in their distribution coefficients between water and ethyl ether. Analogous procedures have been used successfully in conjunction with the microdiffusion method.

Oxidative Procedure for Removal of Pyruvic and Lactic Acids in Acetic Acid Determinations

It is known that pyruvic (4) and lactic acids (5) can be oxidized in alkaline solution to CO_2 and H_2O. We have attempted to remove these substances from solutions of acetic acid by such a procedure.

It was found that alkaline $KMnO_4$, on evaporation in a 100–105°C. oven, completely removed 5 micromoles of lactic acid, using volatile acid recovery as the index of its presence. One micromole, however, was

only partially destroyed. Pyruvic acid was removed completely by the procedure only when lactic acid was also present.

We believe, though it has not been directly demonstrated, that the Mn_2O_3, which is deposited relatively slowly on the flask bottom during lactate oxidation, catalyzes the further oxidation of organic materials in the solution. We have found, in contrast, that MnO or MnO_2 catalyze the formation of acetate from either of these two compounds. Fortunately, manganese ions are removed from filtrates by copper-lime precipitation and thus do not complicate the procedure.

The procedure is of limited usefulness because it will convert certain substances to volatile acids which are not so originally, such as some of the amino acids. The majority of the latter substances tested yielded 0.2–0.3 microequivalent of volatile acid per micromole. Some, however, such as glycine, alanine, tyrosine, and aspartic acid are completely oxidized.

Acetoacetic acid, which does not yield volatile acid when carried through the basic procedure because it is decarboxylated by the strong H_2SO_4, is oxidized almost quantitatively to acetic acid by this procedure. If it is desired to use the oxidative procedure when acetoacetate is present, the latter may first be completely removed by making the filtrate 0.1 *N* with respect to H_2SO_4 and heating in a boiling water bath for 10 min. Sufficient additional alkali must then be added in the oxidative procedure to neutralize the H_2SO_4.

The oxidative procedure reduces the high interference of β-hydroxybutyric acid to about 0.2 microequivalent of volatile acid per micromole. Some of the following acids are oxidized completely and some not at all, but none yields volatile acid when carried through the procedure: citric, isocitric, *cis*-aconitic, α-ketoglutaric, succinic, fumaric, malic and oxaloacetic. Glucose may also be removed in this way, but not interference by adenosine and related substances.

The higher volatile acids are partially oxidized. On the basis of volatile acid recovered, butryric acid is about 50% destroyed and caproic acid 60%.

Some unidentified neutral but volatile product of this procedure causes occasional errors due to alkali creeping out of the steel center cup. Such errors occur only when the amount of volatile acid is very small and can be avoided by making the following two changes in the reagents used in the microdiffusion unit: 1. Substitute 0.005 *M* for 0.0005 *M* acetic acid in making up the 80% H_2SO_4 solution; 2. Substi-

tute 0.04 N $K_2CO_3 \cdot 1.5H_2O$-0.5% KNO_3 for the 0.03 N solution specified

The details of the procedure follow: One ml. of protein-free filtrate is placed in a 50 ml. glass-stoppered Erlenmeyer flask. One-tenth ml. of alkaline lactate and 0.1 ml. of saturated $KMnO_4$ are added in succession. The flask is then tilted gently back and forth 12–15 times to ensure thorough mixing of the solutions (important). Care should be taken to avoid spattering solution high on the flask wall. The open vessel is then placed in the 100–105°C. oven. When the contents have evaporated to dryness, 1 ml. of 0.1 M $SnSO_4$ is added plus about 10 mg. of solid Ag_2SO_4. After gentle shaking 1.0 ml. of 80% H_2SO_4 is added and the analysis carried out by the basic procedure.

Recovery of acetic acid by this procedure from 3 animal tissues is shown in Table V.

TABLE V

Recovery of Acetic Acid from 3 Rabbit Tissues Using Oxidative Procedure

Filtrates were prepared as indicated in Table IV. One ml. aliquots were used for each determination. Volatile acid blank values, expressed as microequivalents/30 mg. of tissue, were as follows: liver 0.03, brain 0.05, and muscle 0.20.

Microequivalents added /30 mg. of tissue	Microequivalents found/30 mg. of tissue		
	Liver	Brain	Skeletal muscle
0.20	0.25	0.17	0.23
0.60	0.60	0.50	0.57
1.20	1.30	1.20	1.17

Colorimetric Method for Acetic Acid

The method of Hutchens and Kass (6) may be applied directly to the acid collected in the microdiffusion center cup, and numerous sources of interference with color development thus avoided. $Ba(NO_3)_2$ equivalent to the $K_2CO_3 \cdot 1.5H_2O$ must be added prior to color development to neutralize the sample and precipitate carbonate.

Deproteinization

Lehninger and Smith, in the development of a specific method for caprylic acid, have determined the conditions required for good re-

covery of fatty acids in protein-free tissue filtrates (7). The details of their procedure as we have used it are indicated in Tables II and IV.

Other Uses of the Apparatus

It is of interest that the ungreased glass stopper of the Erlenmeyer flask allows no significant vapor leakage in a prolonged experiment. Little water is lost during the microdiffusion process despite a water vapor pressure in the flask of about 0.5 atmosphere. We have used the apparatus described here for the determination of ethanol (10–100 γ) and of ammonia (1–10 γ) by published microdiffusion methods (8,9). 100% recoveries were readily obtained.

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Summary

1. A microdiffusion method for determining volatile fatty acids is described. The acids are caused to diffuse in a closed system from an acidified solution to a small cup containing standard alkali. They are subsequently estimated in the cup by a suitable microtitration. The range for the experimental conditions outlined is 0.2–2.0 microequivalents. The average error in determining acetic, propionic and butyric acids is less than 0.03 microequivalent.

2. The method is essentially non-specific. However, individual acids may be distinguished in some instances by their characteristic distribution ratios between water and ethyl ether.

3. A general procedure is described for separating the acids from such interfering materials as lactic and pyruvic acids and others by extraction with ethyl ether.

4. A procedure for the removal of lactic and pyruvic acids by alkaline permanganate oxidation is described, suitable for use in acetic acid determinations under certain circumstances.

5. A simple microburette and microdiffusion unit are described which are of general usefulness in microdiffusion analyses.

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The Biological Degradation of Lignin.[1]
I. Utilization of Lignin by Fungi

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Introduction

Ample evidence exists to indicate that various species of microorganisms are capable of degrading lignin *in situ* in plant materials (1,2,3). Numerous claims have been made in the literature purporting to demonstrate the ability of microorganisms to utilize lignin as a sole carbon source, or purporting to demonstrate the existence of enzymes acting specifically on the lignin molecule (4,5,6). The obvious deterrent to a rational approach to the study of lignin enzymes and of the utilization of lignin by fungi has been the lack of a method for isolating lignin by a procedure which minimizes structural alterations in the lignin molecule during the isolation process. Brauns (7), in 1939, proposed a method for isolating part of the lignin from wood by a process in which no drastic agents (no heat, acid, or alkali) are used. Although the "native lignin" isolated by this process represents only a small part of the lignin in wood, Brauns presents much evidence (7) to indicate the identity, or at least the close similarity, of this material with lignin as it exists in wood. The availability of such a procedure seemed to us to warrant an experimental reexamination of the ability of microorganisms to utilize lignin and of the possible existence of lignin-degrading enzymes.

This paper presents the results of studies on the utilization of native lignin by various fungi; the paper which follows will report the investigations made on a lignin-oxidizing enzyme.

[1] This work was done under contract No. N7 onr 397-4 between the University of Maryland and the Office of Naval Research. The project was initiated at the suggestion of the Prevention of Deterioration Center, National Research Council.

EXPERIMENTAL

Methods and Materials

Cultures. Thirty-five cultures of wood-destroying (white-rot) fungi were employed in this study as listed in Table I. Stock cultures of these organisms were maintained on potato dextrose agar, subcultured at monthly intervals, and stored at 10°C. during the interim.

Preparation of Native Lignin. Native lignin was prepared from freshly cut 12 year old red spruce wood (*Picea rubra*) by the method described by Brauns (8).

TABLE I

Wood-Rotting (White Rot) Fungi Screened for Lignin-Decomposing Activity

Culture number	Genus and species designation	Culture number	Genus and species designation
1	*Polyporus oregonense*	18*	*Fomes pini*
2	*Fomes fomentarius*	19	*Fomes igniarius var. laevigatus*
3*	*Poria subacida*	20	*Fomes pini*
4*	*Poria subacida*	21	*Echinodontium tinctorium*
5	*Pholiota adioposa*	22	*Polyporus anceps*
6	*Corticium galactinum*	23	*Polyporus taugae*
7	*Polyporus valvatus*	24	*Poria weirii*
8	*Ustulina vulgaris*	25	*Fomes annosus*
9	*Fomes geotropus*	26	*Peniophora gigantea*
10	*Stereum sulcatum*	27*	*Fomes annosus*
11	*Lentinus tigrinus*	28*	*Fomes annosus*
12	*Polyporus borealis*	29*	*Fomes pini*
13	*Polyporus abietinus*	30*	*Fomes igniarius*
14	*Poria subacida*	31*	*Ganoderma applanatum*
15	*Collybia velutipes*	32*	*Polyporus circinatus*
16	*Fomes applanatus*	33*	*Fomes igniarius*
17	*Daedelea unicolor*	34*	*Polyporus circinatus*
		35*	*Ganoderma applanatum*

* Cultures received from Dr. Mildred K. Nobles, Ottawa, Canada.

Remaining cultures were obtained from Dr. Ross W. Davidson, Beltsville, Maryland.

Preparation of Lignin for Incorporation into Media. A weighed amount of dry lignin was added to a measured amount of distilled water (2% suspension) and homogenized in a Waring Blendor. This very fine suspension of lignin was then dispensed into 125 ml. flasks and autoclaved for 10 min. at 10 lbs. pressure. This served as the stock lignin for incorporation into media.

The sterilized aqueous suspension of lignin was found to contain a thin layer of dark material as a result of a thermoplastic change during autoclaving. This material

was removed from the flask with aseptic precautions; it amounted to approximately half of the amount of lignin added. The lignin remaining in suspension, on chemical analysis was found to have a methoxyl content of 14.8%, the same as the unheated lignin. The dark material likewise was found to have the same methoxyl content.

For incorporation into media, equal parts of the sterilized lignin and double strength liquid or solid sterile media were combined. This resulted in a final lignin concentration of approximately 0.5% in the media. The stock lignin suspensions ranged in pH from 5.4 to 5.6, and therefore did not alter the pH of the media, which was 5.5.

Some of the final experiments reported here were performed with lignin which was sterilized by exposure to freshly distilled ether. The lignin, after a 3-day contact with ether, was recovered by evaporation of the ether and then, with aseptic precautions, a stock suspension (in water) was prepared, using a Waring Blendor.

The technique employed for incorporation of lignin into solid media consisted of adding 20 ml. of sterilized agar basal medium to a sterile Petri dish or a sterile 125 ml. Erlenmeyer flask. After this layer of medium solidified, it was overlaid with a 5 ml. mixture of the same basal medium containing lignin. This prevented settling out of the lignin, which provided for the mycelial inoculum to be in contact with lignin.

Preparation of Culture Inoculum. In the preparation of mycelial inoculum for test media, it was found necessary to use inocula with minimum carry over of nutrients. Inocula obtained from stock cultures grown on potato dextrose-agar proved to be unsatisfactory. Therefore, a mineral salt medium containing washed agar and inorganic salts was prepared and inoculated from potato dextrose-agar stock cultures. Mycelia produced on this medium were used as inocula for test media, taking precautions to avoid contact with the original portion of inoculum. A third type of inoculum was prepared by inoculating malt extract broth with the original stock-culture inocula contained in 125 ml. Erlenmeyer flasks. These flasks were aerated in a shaking apparatus at incubation temperatures fluctuating between 25 and 30°C. for one week. The mycelial pellets which had formed in these flasks were washed 6 times in sterile distilled water, after which they were suspended in buffered sterile water. These pellets were then used as inoculum.

The following precautions were observed during inoculation of media: Use of uniform amounts of inoculum, floating inoculum on surface of still liquid cultures, and inoculation of central portion of all solid media.

Incubation of Cultures. Throughout the course of this work cultures were incubated at a temperature range of 25–30°C. for periods of 1–4 weeks as indicated in the tables of results. Agar media were dispensed in shallow layers in 125 ml. flasks as well as in Petri dishes. Liquid media were dispensed in 20 ml. amounts in 125 ml. flasks and incubated by both still and shake culture techniques. The shaking apparatus was adjusted to a stroke of 1½″ with 100 three in. excursions/min.

Preparation of Basal Media. At the beginning of this study numerous synthetic and natural media were prepared in an attempt to arrive at an "all or none" growth response to a carbon source. This was complicated by the fact that growth was usually very poor with an inorganic nitrogen source and when organic nitrogen was added, the compound would also serve to a limited degree as a carbon source. The composition of media included in this report are given in the various tables where growth response to lignin is recorded.

All media were adjusted to pH 5.5 and then sterilized at 15 lbs. pressure (121°C.)

for 15 min. Glucose and thiamine were sterilized separately by filtering through a sintered glass filter, after which they were added aseptically to the already sterilized medium according to the concentration desired. The mineral supplement employed in all media was Hoagland's A–Z mixture (9).

Ten ml. of a 1% sterilized aqueous suspension of lignin were aseptically added to equal amounts of double strength concentrations of pre-sterilized basal media, when a lignin-containing medium was desired.

Measurement of Growth Response

a. Solid Media. Measurement of the diameter of giant mycelial colonies were recorded in mm. No attempt was made to measure the comparative thickness of the mycelial mats.

b. Liquid Media. Measurement of mycelial growth in liquid media was made in 2 ways. (1) Comparative visual observations recorded as a series of +'s or −'s if no growth was evident; (2) weighing of the mycelial mat, by use of the following technique: Liquid culture media left in the flasks after incubation was carefully poured off, leaving only the mycelia in the culture flasks. To this flask was then added 25 ml. of dioxane, after which the flask was placed on a shaking apparatus and shaken for 8 hr. to remove any unutilized, adsorbed lignin which may have adhered to the mycelium during incubation. After shaking, the mycelial-dioxane mixture was filtered through an asbestos pad contained in a tared procelain Gooch crucible, washed again with 25 ml. of dioxane, and then dried at 55°C. until constant weight was obtained. This procedure was also applied to the mycelia growing in the control flasks which contained no lignin.

Results

Screening Experiments on Liquid and Solid Media

During initial attempts to screen the 35 white rot fungi cultures for utilization of lignin, it was found that a clear-cut "all or none" growth response could not be obtained when lignin was the limiting carbon source in the medium. Some of the difficulties encountered were as follows:

On agar media, most of the cultures grew to some extent on the basal medium without added carbohydrate (Table II). This may have been due to carry over of some nutrients with the inoculum, size of inoculum, which was variable, *etc.* In liquid media, an inorganic source of nitrogen, such as NH_4NO_3 or $(NH_4)_2SO_4$, did not prove satisfactory. Supplementing such inorganic nitrogen in the medium with amino acids or casein hydrolyzate resulted in media upon which the fungi would grow to some degree without added carbohydrate. As a consequence, numerous solid and liquid media were prepared and all 35 cultures were tested. Each medium was prepared in duplicate sets, one with and one without lignin.

TABLE II

Results of Screening Experiments to Demonstrate Lignin Utilization by White Rot Fungi on Solid and Liquid Media

Culture number	Growth on solid media after 2 weeks incubation		Growth on liquid media after 4 weeks incubation	
	Basal[a] medium	Basal medium plus 0.5% lignin	Basal[b] medium	Basal medium plus 0.5% lignin
	mm.[c]	*mm.*[c]		
1	20	10	–[d]	–[d]
2	45	90	+	2+
3	35	60	+	2+
4	15	30	+	2+
5	25	60	–	–
6	30	55	±	+
7	10	–	2+	2+
8	10	65	+	+
9	90	90	+	2+
10	15	–	–	–
11	90	90	+	2+
12	35	20	–	–
13	15	90	±	+
14	25	90	±	+
15	45	90	+	2+
16	25	45	±	+
17	25	90	±	2+
18	30	5	–	–
19	25	10	+	+
20	5	30	+	+
21	10	–	–	–
22	45	60	+	2+
23	55	10	+	+
24	15	25	±	+
25	30	90	±	+
26	60	90	±	2+
27	25	5	–	–
28	45	10	–	–
29	50	10	–	–
30	40	15	–	–
31	35	15	–	–
32	12	–	–	–
33	30	75	+	2+
34	5	–	–	–
35	5	35	+	2+

[a] Basal medium:

NH_4NO_3	0.5%	Agar	2.00%
KH_2PO_4	0.15%	Thiamine (HCl)	0.001%
$MgSO_4 \cdot 7H_2O$	0.05%	Mineral supplement	1 ml.
$CaCO_3$	0.02%	Water (distilled)	1 l.

[b] Same as above, but without agar incorporated.

[c] Diameter of giant colony in mm.

[d] Reading of – indicates no growth; ±, doubtful growth response; +, small mycelial mat; 2+, half the liquid surface covered with mycelial mat.

Examples of the type of response obtained in solid and liquid media containing inorganic nitrogen, with and without lignin, are presented in Table II. The cultures which seemed to produce greater amounts of mycelia on the solid medium containing lignin were as follows: No. 3, *Poria subacida;* No. 4, *Poria subacida;* No. 8, *Ustulina vulgaris;* No. 13, *Polyporus abietinus*; No. 14, *Poria subacida;* No. 15, *Collybia velutipes;* No. 17, *Daedalea unicolor;* No. 25, *Fomes annosus;* No. 26 *Peniophora gigantea;* No. 33, *Fomes igniarius*; and No. 35 *Ganoderma applanatum.*

Several of the cultures gave indications of being inhibited by the presence of lignin, in which instance they produced no evidence of growth on the basal medium containing lignin. These cultures were as follows: No. 7, *Polyporus valvatus;* No. 10, *Stereum sulcatum;* No. 21, *Echinodontium tinctorium;* No. 32, *Polyporus circinatus;* and No. 34, *Polyporus circinatus.* Growth in the liquid medium described in Table II was irregular, and, in those cases where growth did occur, it was not very abundant. It was, however, significant to note that cultures No. 6, *Corticium galactinum*, No. 13, *Polyporus abietinus*, No. 14, *Poria subacida*, No. 16, *Fomes applanatus*, No. 17, *Daedalea unicolor*, No. 24, *Poria weirii*, and No. 25, *Fomes annosus*, presented evidence of growth in the medium containing lignin, but gave a doubtful growth response in the medium minus lignin.

Although the results presented in Table II were suggestive of growth response to lignin by several of the fungi employed, the results were not interpreted as definitive. Repeated experiments following the scheme presented in this table gave variable results so that a consistent, unequivocal response to lignin could not be attained.

Adaptation Technique for Lignin Utilization

As a consequence of the results obtained in the screening experiments described above, an attempt was made to stimulate utilization of lignin by a process of adaptation. For this purpose, a basal medium of the composition indicated in Table III which contained 0.1% asparagine as a supplementary source of nitrogen was employed. Asparagine in conjunction with $(NH_4)NO_3$ was found to provide a more suitable source of nitrogen than $(NH_4)NO_3$ alone.

For these experiments, the following cultures, which previously had exhibited stimulation of growth in the presence of lignin, were employed: No. 9, *Fomes geotropus;* No. 11, *Lentinus tigrinus*; No. 13, *Polyporus abietinus;* No. 14, *Poria subacida;* No. 17, *Daedalea unicolor;* and No. 26, *Peniophora gigantea.* Each of these cultures was

TABLE III

Adaptation of White Rot Fungi to Lignin Utilization

Amount of mycelial growth in mg. of several white rot fungi serially subcultured after 3 week incubation periods in a basal medium with decreasing glucose concentration and constant lignin concentration.

Still Cultures

Culture number	Basal medium[a] plus glucose					Basal medium plus glucose and 0.5% lignin				
	Per cent glucose and serial subculture[b]					Per cent glucose and serial subculture				
	0.1% 1st	.05% 2nd	.025% 3rd	.01% 4th	0% 5th	0.1% 1st	.05% 2nd	.025% 3rd	.01% 4th	0% 5th
9	15[c]	17	15	10	4	18	20	15	11	3
11	20	22	18	14	5	25	24	21	16	6
13	10	8	5	4	1	20	22	25	26	29
14	14	11	9	6	4	37	39	35	31	33
17	30	21	15	11	7	50	40	35	28	15
26	20	15	10	8	3	22	17	14	12	4

Shake Cultures

9	12	15	14	12	6	22	18	16	13	6
11	24	20	18	17	9	30	26	24	19	11
13	13	10	7	5	2	23	25	29	30	31
14	28	8	5	2	3	22	21	23	25	28
17	43	32	21	13	10	52	41	25	18	13
26	15	13	11	10	4	25	19	17	14	6

[a] Basal medium:

NH_4NO_3	0.5%
L-(+)-Asparagine	0.1%
KH_2PO_4	0.15%
$MgSO_4 \cdot 7H_2O$	0.05%
$CaCO_3$	0.02%
Thiamine (HCl)	0.001%
Mineral supplement	1 ml.
Water (distilled) to	1 l.

[b] Cultures incubated for 3 weeks, whereupon a small piece of mycelium was transferred to a similar medium containing less glucose. This process involved 5 serial cultures, the last medium containing no glucose.

[c] Figures represent weight of mycelia in mg.

inoculated into 2 flasks of the basal medium, one of which contained glucose (0.1%) and the other both lignin (0.5%) and glucose (0.1%) as indicated in Table III. Duplicate sets were inoculated, one set incubated in still culture and the other by shake culture. After an incubation period of 3 weeks, the above cultures were subcultured into new flasks containing the same media except for a decrease in the glucose content as is indicated in Table III. This process of subculturing into media in which the glucose concentration had been diminished to half of its preceding concentration was repeated at 3-week intervals until the glucose concentration had been reduced to 0, after which time lignin remained as the limiting source of carbon in one of the media.

The pH of the media and dry weight of the mycelium was determined for each culture flask after each 3-week incubation period by the technique described under "Materials and Methods." The mycelial weight determinations are recorded in Table III. The pH values are not recorded, as no significant trend was noted.

From the results presented in Table III, it can be seen that cultures No. 13, *Polyporus abietinus*, and No. 14, *Poria subacida*, showed evidence of increased ability to utilize lignin during the course of 5 serial subcultures in the presence of lignin. Growth in the medium with glucose alone decreased with each decrease in glucose concentration. However, in the medium containing a constant amount of lignin (0.5%), growth actually showed some increase, even though the glucose content was decreased and finally omitted entirely.

Cultures No. 9, *Fomes geotropus*, No. 11, *Lentinus tigrinus*, and No. 26, *Peniophora gigantea*, showed no evidence of adaptation to lignin utilization under the conditions of the experiment. After the fourth serial subculture, growth was practically negligible in both the medium with 0.01% glucose and that containing 0.01% glucose and 0.5% lignin. Culture No. 17, *Daedalea unicolor*, showed little if any increase in ability to utilize lignin.

It was also evident from the results presented in Table III that the total mycelial yield was higher for all cultures in the media containing glucose (at the higher concentrations) plus lignin than in the corresponding medium containing glucose alone.

During the course of the adaptation experiments, subcultures were made from the liquid media onto 4 solid media of the same basic composition. This was done to obtain additional evidence for the degree of growth response to lignin after the culture had been in contact with lignin for various periods of time. It was found that cultures No. 13 *Polyporus abietinus*, and No. 14, *Poria subacida*, produced a large mycelial mat on the lignin containing agar while the other 4 cultures grew very sparsely on the same medium.

Comparisons of the response of cultures No. 13, *Polyporus abietinus*, and No. 14, *Poria subacida* (adapted and unadapted) to lignin were made by inoculating each of these cultures onto a solid medium which contained lignin as the limiting source of carbon. It was clearly demonstrated that the cultures which had been grown in the presence of lignin (inoculum taken from 3rd serial subculture—Table III) had developed a considerable ability for utilization of lignin, as evidenced by the large mat of mycelium which developed on the lignin medium. The media inoculated with the unadapted culture (inoculum taken from growth on potato dextrose-agar slant) produced very sparse growth.

Response to Chemically Sterilized Lignin and Autoclaved Lignin

It has been stated that the lignin for incorporation into the medium was sterilized in an aqueous suspension by autoclaving. However, it was possible to sterilize the lignin by exposure to ether as described under "Materials and Methods." Growth response to the chemically sterilized lignin was the same as was observed with autoclaved lignin.

Specificity of Growth Response to Lignin

To eliminate the possibility that the growth response observed and interpreted as lignin utilization was not due to impurities, the lignin was subjected to further purification, and growth response determined on the resulting product. The original lignin was repurified by dissolving in dioxane, reprecipitated in ether, and washed in ether, benzene, and petroleum ether. This process was repeated 10 times. The final product was designated repurified native lignin. The original and repurified lignin were then separately incorporated into solid media as the sole source of carbon and inoculated with cultures No. 13, *Polyporus abietinus*, and No. 14, *Poria subacida*. The inoculum was taken from the 3rd serial subculture flasks containing lignin (Table III). It was observed that growth response did not differ on either type of lignin. It was, therefore, concluded that the stimulation of growth was due specifically to the lignin.

SUMMARY

Thirty-five cultures of white rot fungi were screened for their ability to utilize native lignin. Under the experimental conditions described, many of these cultures exhibited growth response when lignin was the

limiting source of carbon in the medium. However, the results from these screening experiments could not be clearly evaluated because of variations in growth on duplicate determinations and other irregularities in growth response.

As a consequence, 6 of the cultures were selected and an attempt made to adapt them to lignin utilization by growing the cultures for a prolonged period in a lignin-glucose medium, gradually diminishing the glucose content until it reached zero. By this approach, it was possible to obtain two cultures, No. 13, *Polyporus abietinus*, and No. 14, *Poria subacida*, which consistently produced an appreciable amount of growth on a solid or liquid medium in which lignin was the limiting source of carbon. Both of these media were of chemically defined composition except for the agar in the solid medium.

The availability of such a technic for rapid growth of fungi on a synthetic medium containing lignin provides for a more rigorous and rational approach to the mechanism of biological degradation of lignin than has been hitherto possible.

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Studies on the Permeability of Erthrocytes. I. The Relationship between Cholinesterase Activity and Permeability of Dog Erthrocytes [1]

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Introduction

Evidence that the internal ionic composition of the erythrocyte is regulated by metabolic processes has been supplied by a number of workers (9,11,20,25,26,45). Wilbrandt observed that the addition of NaF or sodium iodoacetate is followed by a large loss of potassium from the mammalian red cell. This effect he attributed to the inhibitory action of these drugs on glycolysis. Le Fevre provided evidence that, in the case of hemolysis of erythrocytes by glycerol or glucose, some phosphorylation mechanism is involved which is sensitive to sulfhydryl agents. Apart from these suggestions, no specific metabolic processes seem to have been considered.

In some experiments on the matabolic effects of methadon,[3,4] we observed that, following the intravenous administration of this drug, dogs occasionally showed a hemoglobinuria and frequently an increase in red cell fragility (35). In experiments *in vitro* methadon also caused hemolysis of dog erythrocytes. We had previously found that methadon inhibited the glycolysis of glucose by brain but had no effect on the glycolysis of glycogen or of phosphorylated hexoses (18). The permeability changes in erythrocytes produced by methadon might be explained by the inhibitory action of this drug on glycolysis as postulated

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[2] Research Fellow, U. S. Public Health Service.

[3] Kindly supplied by Mallinckrodt Chemical Works.

[4] Methadon or amidone, 2-dimethylamino-4,4-diphenylheptanone-5-hydrochloride.

by Wilbrandt for the action of NaF and sodium iodoacetate. However, as we used washed cells, it is unlikely that there would be appreciable glucose in the medium. Methadon also inhibited cholinesterase (13,14, 19) as do NaF and sodium iodoacetate (30). Red cells are a rich source of cholinesterase (2), which is situated in the cell membrane or stroma (7,33). It seemed possible, on the basis of certain considerations to be described later, that the acetylcholine-cholinesterase system instead of, or in addition to, the enzymes involved in glycolysis might be concerned with permeability of the red cell. To test this hypothesis further, the effect of physostigmine, a specific inhibitor of cholinesterase, on dog erythrocytes was investigated.

Methods

Cholinesterase activity was determined both manometrically, using the Warburg technique, and by titration to constant pH with $N/200$ NaOH, the pH being determined by use of the Coleman pH meter. Both determinations were carried out at 37°C. in the media described in the tables.

Freshly drawn blood was defibrinated, centrifuged, and the red cells washed with isotonic buffer or saline. In some experiments 0.5 cc. of a 50% suspension, in others, 0.2 cc. of packed red cells were used.

The buffers used were the following: Ringer-Krebs bicarbonate buffer (24); bicarbonate saline consisting of 100 parts isotonic NaCl or KCl and 21 parts isotonic $NaHCO_3$ or $KHCO_3$; phosphate saline buffer consisting of 100 parts isotonic NaCl or KCl and 21 parts isotonic phosphate (mixtures of dibasic and monobasic phosphates in the proportions specified by Sørensen (38) to give the desired pH).

The concentration of acetylcholine used in the experiments was 10^{-2} M. The concentration of methadon was 8.7×10^{-4} M, and that of physostigmine varied between 7.2×10^{-4} M and 3.6×10^{-8} M, as indicated in the tables and graphs. It was assumed that, at pH 7 and higher, acetylcholine bromide and methadon hydrochloride were completely ionized, and on the basis of these assumptions stock solutions which were isotonic with plasma were made. Physostigmine was used in the form of the free base and the stock solution was also made isotonic with plasma.

In some experiments the fragility of red cells was determined by Sanford's method (37), in which the red cell suspension was added to solutions of varying concentrations of NaCl. In other experiments, the degree of hemolysis was determined by removing samples from the experimental flask at intervals throughout the experiment, centrifuging, and determining the hemoglobin in the supernatant fluid by use of a photoelectric colorimeter.

Results

In the investigation described, the blood of 7 different dogs was used. The results of only a few experiments, which are representative of a much larger number, are presented. Isotonic solutions were used throughout.

In preliminary experiments, the effects of methadon and of physostigmine on red cell cholinesterase activity were determined manometrically in a sodium, potassium, or Ringer-Krebs' bicarbonate buffer at pH 7.4 with 95% N_2–5% CO_2 as the gas phase. At the end of the experimental period, fragility tests by Sanford's method showed that methadon always increased fragility while physostigmine had little, or a variable, effect (Table I). It was observed, however, that, if the experimental flasks were allowed to stand at room temperature for 2–3 hr., complete hemolysis of the physostigmine-treated cells frequently occurred, while hemolysis was considerably less in the methadon-treated suspension.

TABLE I

Effect of Methadon and of Physostigmine on Dog Erythrocyte Cholinesterase

Each Warburg vessel contained 0.5 cc. of a 50% suspension of erythrocytes and acetylcholine $M/100$ in a final volume of 2 cc. The media were: 1. Ringer-Krebs bicarbonate buffer; 2. 100 parts isotonic KCl, 21 parts isotonic $KHCO_3$.

Duration	Medium	Drug	Conc. of drug	mm.³ CO_2 evolved			Per cent NaCl in which hemolysis began and was complete			
							Control		With drug	
				Control	With drug	Inhibition	Begun	Complete	Begun	Complete
min.			*M*			*per cent*				
95	1	Methadon	8.7×10^{-4}	238	197	17	.54	.40	.66	.52
95	2	Methadon	8.7×10^{-4}	267	238	11	.66	.40	.66	.58
60	1	Methadon	8.7×10^{-4}	159	104	35	.44	.34	.66	.44
50	1	Physostigmine	7×10^{-4}	229	77	66	.52	.34	.52	.34
60	1	Physostigmine	7×10^{-5}	257	28	89	.52	.46	.54	.38
60	1	Physostigmine	7×10^{-6}	257	130	49	.52	.46	.50	.46
60	1	Physostigmine	7×10^{-8}	257	210	19	.52	.46	.52	.44

These seemingly contradictory results might be attributed (1) to a discrepancy between the results of the Sanford test for fragility and the degree of hemolysis actually occurring in the experimental medium, or (2) to the increased pH caused by the escape of CO_2 from the medium, or (3) to physostigmine having a delayed effect but causing rapid hemolysis after the onset. To test these possibilities, large scale experiments were carried out in Erlenmeyer flasks, and 1 cc. aliquots were removed at intervals, diluted with isotonic solutions, centrifuged, and the free hemoglobin determined in the supernatant fluid colorimetrically. It was found that fragility, as determined by the Sanford method, did not necessarily parallel the hemolysis produced in the experimental flask. When KCl was substituted for NaCl in the Sanford test, different results were obtained. It proved more satisfactory to determine the hemoglobin directly after centrifugation of a sample of the experimental suspension.

FIGURE I

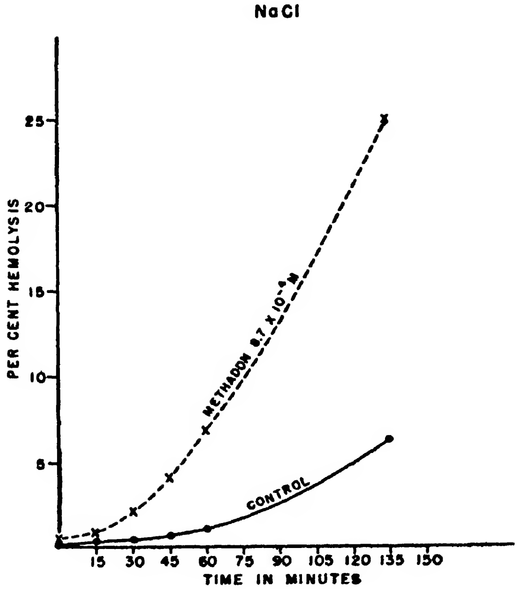


FIGURE 2

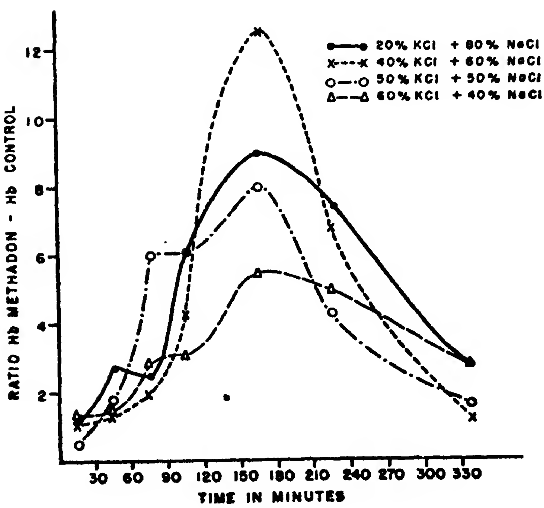


FIGURE 3

KCl

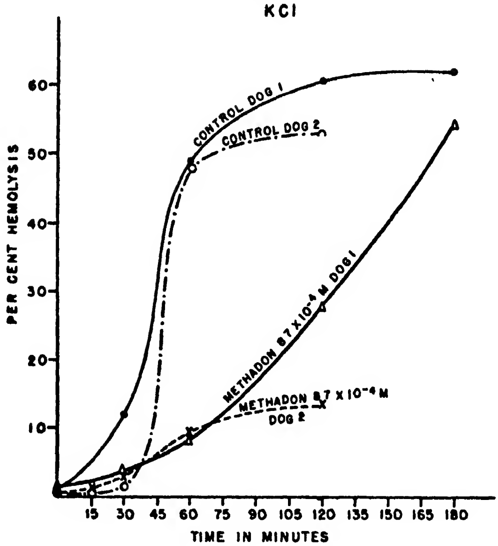


FIGURE 4

10% NaCl + 90% KCl

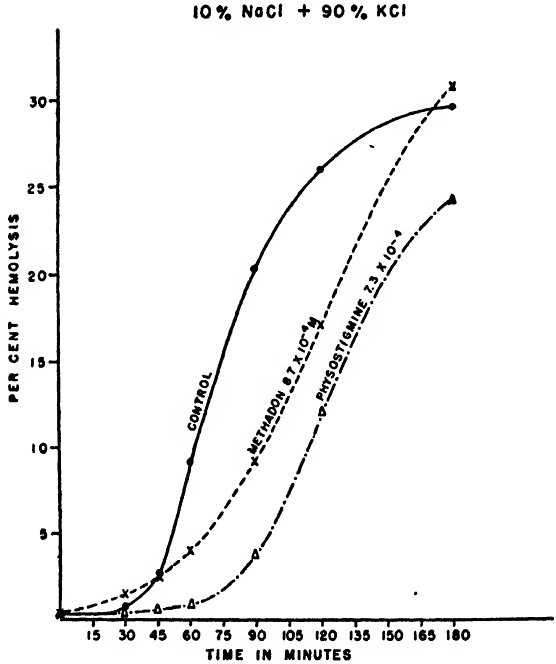


FIGURE 5

$5\%\ NaHCO_3 + 95\%\ KHCO_3$

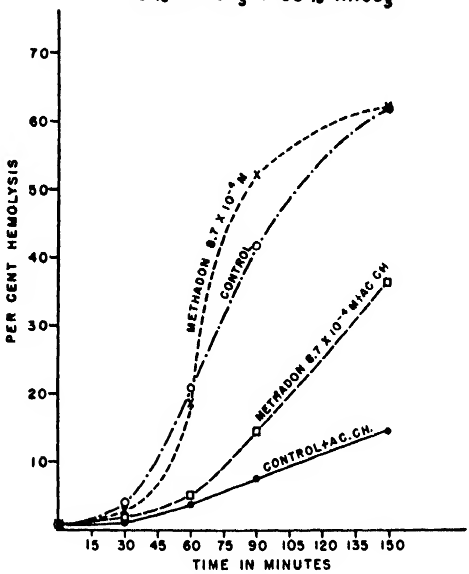


FIGURE 6

$5\%\ NaHCO_3 + 95\%\ KHCO_3$

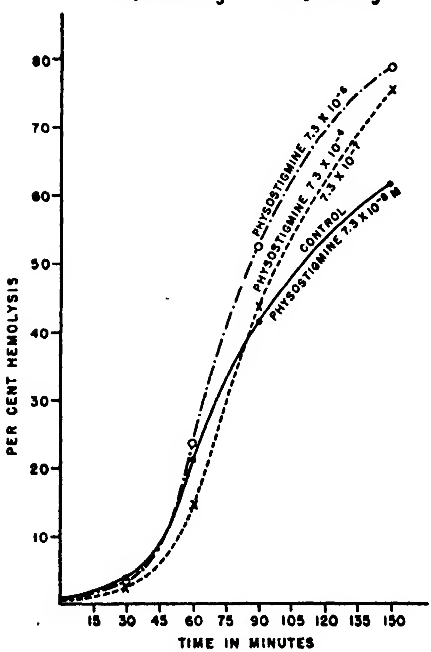


FIGURE 7

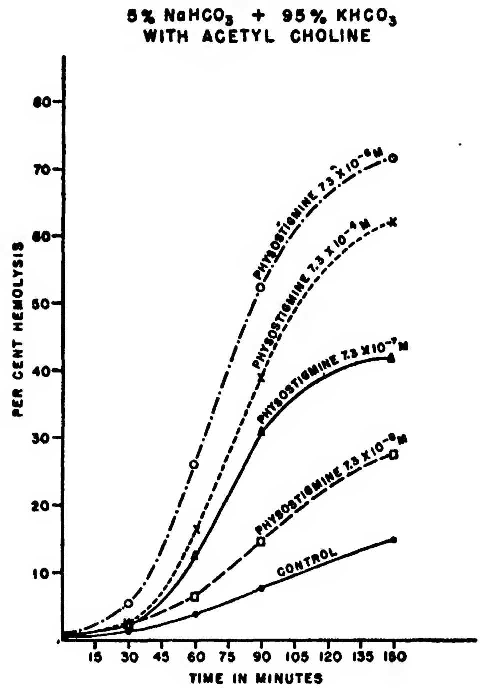
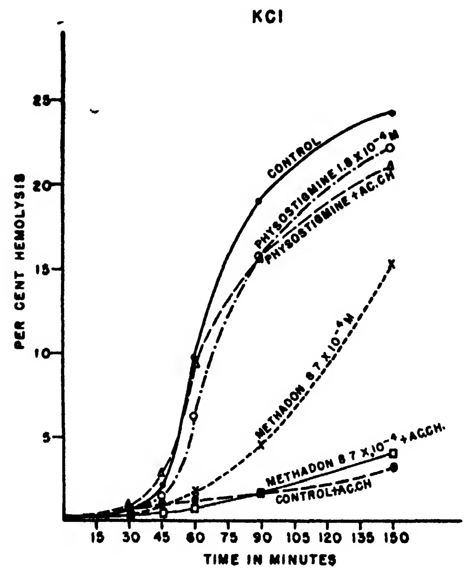


FIGURE 8



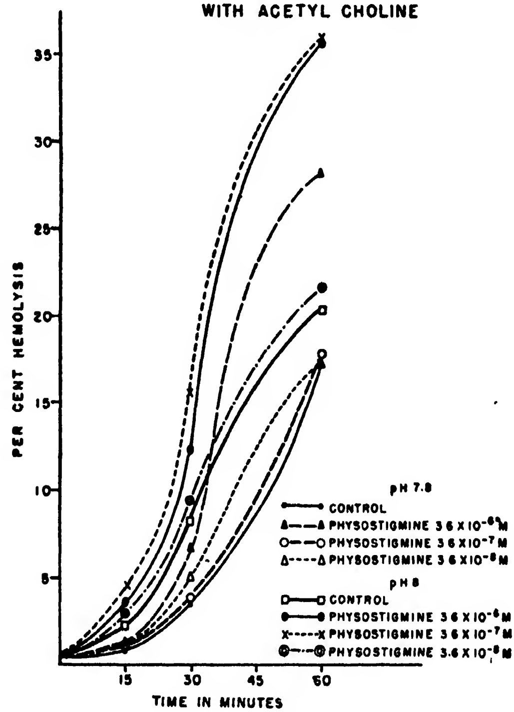
The factors which influenced the changes in permeability produced by physostigmine and methadon to the greatest extent were found to be the ionic composition of the medium, the pH, and the presence or absence of acetylcholine.

Effect of Cations and of Acetylcholine

Na^+, K^+-Methadon. In NaCl, and in mixtures of isotonic solutions of NaCl and KCl in proportions varying between 90% and 10% NaCl, methadon increased the hemolysis of dog erythrocytes (Fig. 1). The

FIGURE 9

95% K PHOSPHATE SALINE + 5% NaCl
pH 7.8 AND 8
WITH ACETYL CHOLINE



FIGS. 1–9. One cc. packed erythrocytes in saline or buffer, either alone or with acetylcholine or the cholinesterase inhibitor, or with both of these drugs, were incubated in a water bath at 37°C. Final volume 10 cc. Final concentration of acetylcholine 0.01 *M*. Concentration of cholinesterase inhibitors is indicated on the graphs. Symbols on the graphs denote isotonic solutions, *e.g.*, 90% KCl–10% NaCl signifies isotonic solutions of KCl and NaCl in the proportion 9:1.

TABLE II

Effect of Na and of K Ions on the Inhibition by Physostigmine of Cholinesterase

Buffers consisting of either 100 parts isotonic NaCl and 21 parts isotonic $NaHCO_3$ or KCl and $KHCO_3$ in the same proportions, or mixtures of these two buffers were used. The concentration of acetylcholine was 0.01 *M*.

Expt.	Duration	Conc. of		Conc. of physostigmine	mm.³ CO_2 evolved		Effect
		Na	K		Control	With drug	
	min.	*per cent*	*per cent*	*M*			*per cent*
164	90	95	5	7×10^{-6}	223	86	−61
		95	5	7×10^{-7}	223	223	0
		95	5	7×10^{-8}	223	223	0
		5	95	7×10^{-6}	256	79	−76
		5	95	7×10^{-7}	256	151	−41
		5	95	7×10^{-8}	256	223	−13
162	50	100	–	7×10^{-6}	178	45	−75
		95	5	7×10^{-6}	151	39	−74
		–	100	7×10^{-6}	151	0	−100
176	50	100	–	3.6×10^{-6}	129	24	−82
				3.6×10^{-7}		66	−49
		50	50	3.6×10^{-6}	100	35	−65
				3.6×10^{-7}		82	−17
178	60	100	–	3.6×10^{-8}	115	149	+29.6
		–	100	3.6×10^{-8}	124	100	−20
		–	100	3.6×10^{-9}	124	121	0

composition of the medium in which maximum hemolysis occurred varied somewhat in different experiments, but the time at which the maximum effect of methadon was produced was fairly constant. In 3 experiments, in which bloods of 3 different dogs were used, the times at which maximum hemolysis occurred were 165, 165 and 180 min. from the start of the experiment (Fig. 2). In KCl solutions, and occasionally in solutions containing 90% KCl and 10% NaCl, methadon produced an increase in resistance of dog erythrocytes (Figs. 3,4).

Na⁺, K⁺-Physostigmine. In NaCl solution, physostigmine caused only slight increases in hemolysis, and these effects occurred late in the experiment (around 150–200 min.). As the concentration of K was increased, the permeability of the physostigmine-treated suspension

deviated more and more from the control, and in KCl as well as in 5% or 10% NaCl in KCl, the physostigmine-treated erythrocytes were more resistant than were those treated with methadon (Fig. 4).

Acetylcholine. In KCl, in bicarbonate buffer, or in phosphate-saline buffer, the addition of acetylcholine increased the resistance of red cells and at the same time increased the differences in the degrees of hemolysis between the control and the drug-treated cells (Figs. 5,6,7,8). Under these conditions, physostigmine caused increased hemolysis of erythrocytes, which is in contrast to the effect produced by this drug in KCl without acetylcholine, where the cells were made more resistant (Fig. 8). In bicarbonate buffer, physostigmine in concentrations between 7.3×10^{-4} *M* and 7.3×10^{-7} *M* produced a maximum relative hemolytic rate in about 90 min.

Effect of pH

While methadon produced changes in resistance of red cells over a wide range of Na:K concentrations, the physostigmine effect, in experiments of short duration, occurred most markedly in a medium in which the proportion of K was high compared with that of Na. Likewise, while methadon was active over a wide range of pH the activity of

TABLE III

Effect of pH on Physostigmine Inhibition of Cholinesterase

2 cc. packed erythrocytes, 2 cc. 10% acetylcholine and 22 cc. isotonic NaCl or KCl were incubated at 37°C., and titrated with 0.02 *N* NaOH. Physostigmine 9×10^{-5} *M*.

Medium	Duration of expt.	pH	cc. NaOH			Inhibition
			Control	With physostigmine	Spontaneous hydrolysis of acetylcholine	
	min.					*per cent*
NaCl	20	7.4	1.86	1.20	.41	46
		8.0	3.10	1.23	.45	72
NaCl	20	7.4	1.55	1.0	.41	48
		8.0	4.10	1.7	.45	66
KCl	10	7.2	0.87	.70	.18	25
		8.0	1.13	.50	.23	70

physostigmine increased quite markedly as the pH was increased (Fig. 9).

At pH values of 7.8–8 physostigmine caused an increase in hemolysis in contrast to its effect in KCl, in which it produced an increased resistance (*cf.* Figs. 9,4). Thus, an increased resistance produced by physostigmine may be changed to an increased hemolysis either by increasing the pH or by the addition of acetylcholine.

The increased effect of physostigmine in media of high pH and of high K content parallels the increased activity of cholinesterase under these conditions (Fig. 4) (Tables II, III). Also these conditions appear to be optimum for the inhibitory action of physostigmine (Tables II, III).

In low concentrations, the inhibitory effect of physostigmine on cholinesterase and its effect on permeability wear off earlier than when higher concentrations are used. This may be due to destruction of the drug.

Discussion

The experiments described show that, under certain conditions, both methadon and physostigmine will produce changes in permeability of dog erythrocytes. As already stated, methadon inhibits the glycolysis of glucose in addition to cholinesterase activity, but, under the conditions of our experiments, in which washed cells were used, it is unlikely that there would be significant quantities of free glucose in the medium. Any carbohydrate inside the cell would probably be glycogen or phosphorylated hexose, the metabolism of which we found to be unaffected by methadon. Effects of methadon on cell permeability under these conditions would not likely be connected with its action on carbohydrate metabolism. NaF and sodium iodoacetate, which Wilbrandt (44,45) showed could produce changes in permeability of erythrocytes, are both glycolytic inhibitors and inhibitors of cholinesterase. While glycolysis is sensitive to lower concentrations of NaF and sodium iodoacetate than is cholinesterase, the concentrations of these drugs required to produce changes in permeability, as found by Wilbrandt, were of the same order of magnitude as those which produce significant inhibitions of cholinesterase activity. Phosphoglyceraldehyde dehydrogenase activity is inhibited by iodoacetate in concentrations of $M/3000$ (1). However, Wilbrandt found that changes in permeability of red cells occurred only when considerably higher con-

centrations ($M/500$–$M/100$) were used. According to Nachmansohn and Lederer (30), cholinesterase is inhibited by iodoacetate in concentrations of $M/100$. Wilbrandt found that NaF in concentrations of around $M/200$–$M/145$ inhibited glycolysis but had little effect on permeability, while concentrations of $M/145$–$M/72$ produced changes in permeability. Massart and Dufait (29) used concentrations of $M/50$ and $M/100$ NaF to inhibit cholinesterase 60 and 30%, respectively. It would thus seem possible that cholinesterase activity as well as glycolytic activity might be inhibited under Wilbrandt's experimental conditions. It is also possible that specific inhibitors of glycolysis could affect permeability by virtue of their inhibiting the formation of adenosine triphosphate and of pyruvate, both of which may be used in the synthesis of acetylcholine (27,28,32). Physostigmine is considered a specific inhibitor of cholinesterase. Deutsch and Raper (12) found that, under certain conditions, physostigmine with acetylcholine increased the carbohydrate metabolism of slices of the cat's submaxillary gland *in vitro* but had no inhibitory action. If similar changes in permeability produced by both methadon and physostigmine are due to a single metabolic disturbance, it would seem that, of the known effects, the action on cholinesterase would provide the best explanation.

Both methadon and physostigmine produced increased fragility and increased resistance of dog erythrocytes depending on the medium. In KCl both produced increases in resistance. In NaCl methadon caused increased hemolysis while physostigmine had little effect on experiments of short duration. Whether increased fragility or increased resistance results, may also depend on the composition of the erythrocytes. The exact interpretation of these results, however, awaits experiments in which the action of physostigmine and methadon on the prolytic changes are determined.

Methadon was active in causing hemolysis over a wider range of Na–K concentrations and of pH than was physostigmine, but in a medium of a high K concentration and a pH of about 8, physostigmine was a powerful hemolytic agent. The hemolytic activity of physostigmine was increased by acetylcholine. It should be noted that the optimum conditions for hemolysis by physostigmine paralleled the optimum conditions for cholinesterase activity and, under these conditions, physostigmine also exerted its maximum inhibitory action (Tables II, III). The optimum conditions for the action of methadon on cholinesterase are being investigated and will be reported later.

It might be of interest to note that certain other compounds which, although they undoubtedly have other actions, are known to be inhibitors of cholinesterase activity also influence the permeability of erythrocytes. One of these drugs, namely morphine, which is a hemolytic agent *in vitro* (5,23,34), an inhibitor of cholinesterase *in vitro* (4,46,47), and which has been reported to cause an anemia *in vivo*, has been investigated for its effect on glycolysis by brain in this laboratory (18). It was found that, in concentrations up to 5.6×10^{-3} *M*, it either had no effect or accelerated glycolysis. This being the case, its hemolytic action cannot be explained by its inhibiting the anaerobic metabolism of glucose. Lead, which produces a hemolytic anemia (6,22), causes marked changes of permeability of red cells *in vitro* (21) and is also a potent inhibitor of cholinesterase (16). Tetanus toxin, which produces a secondary (15) anemia in rabbits, is also an inhibitor of cholinesterase (17).

In pernicious anemia, red cells have been reported to show an abnormal permeability (3). The erythrocyte cholinesterase is also low in this condition (36).

Davis (10) reported the production of hyperchromic anemia in dogs following the administration of acetylcholine with physostigmine.

There is some evidence that the acetylcholine-cholinesterase system may be concerned with the permeability of tissues other than red cells. Swan and Hart (40) showed that physostigmine, carbaminoyl choline and mecholyl increased the permeability of the blood-aqueous humor barrier to dyes, and Stocker (39) observed that miotics increased the permeability of the blood-aqueous barrier when instilled into the eye. Cumings (8) reported changes in serum and muscle potassium after the administration of prostigmine, an inhibitor of cholinesterase, to myasthenic patients. Thompson and Tice (41) observed that prostigmine produced a decrease in serum potassium in dogs and cats and an elevation of muscle potassium in rats.

That the acetylcholine-cholinesterase system is concerned with changes in permeability in the nerve cell may be postulated from the work of von Muralt (42) and of Nachmansohn and collaborators (30,31,32), who have provided considerable evidence that the passage of an impulse along a nerve is dependent on the rapid formation or liberation and removal of acetylcholine within the cell. This being the case, the change in permeability which accompanies the change in potential could also be explained by the formation and removal of acetylcholine.

Welch (43), in a discussion of the function of acetylcholine and cholinesterase in nerve transmission, has suggested that acetylcholine may be a coenzyme which is concerned with the activity of an enzyme in the cell membrane, and that the role of this enzyme is to alter excitability of the cell through changes in membrane polarity. Such an idea might also be applicable to red cell permeability.

Investigations of the effect of cholinesterase inhibitors on the prolytic exchange of erythrocytes are being undertaken.

Summary

1. Evidence is presented that changes in the permeability of the erythrocyte may be effected by inhibition of the activity of the acetylcholine-cholinesterase system which is situated in the cell membrane.

2. Both methadon and physostigmine, which are inhibitors of cholinesterase, produce changes in permeability of dog erythrocytes.

3. The effect of these drugs on permeability is influenced by the sodium and potassium content of the medium, the pH, and the presence of acetylcholine.

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Studies on Cell Enzyme Systems. II. Evidence for Enzyme-Substrate Complex Formation in the Reaction of *Cypridina* Luciferin and Luciferase [1]

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Introduction

Most studies on enzyme kinetics and equilibria have been carried out with hydrolytic enzymes by measureing the amount of substrate disappearing or reaction products which appear during given intervals of time. Oxidative enzyme reactions have received less attention, despite their importance as energy-controlling processes, largely because the process frequently occurs in a complex series of steps.

The enzyme-catalyzed oxidative bioluminescent reactions, which are probably in some forms related to cellular respiratory processes, are unique in that the intensity of the emitted light is a direct, instantaneous measure of the velocity of the underlying reaction. Moreover, luminescence measurements are easily made and can, if necessary, be automatically recorded. The ostracod crustacean, *Cypridina hilgendorfii*, is one of a few luminous organisms from which the enzyme and substrate of the reaction can be readily extracted and separated (10). If the animals are dried when collected and kept dry, the luciferin and luciferase remain stable apparently indefinitely. Furthermore, they are stored in such quantity in this animal that they can be subjected to purification procedures. It is thus possible to study the reaction *in vitro*, using relatively pure compounds and controlled conditions. This reduces the effects of unknown complicating factors.

The theory of an intermediate enzyme-substrate complex, derived and tested by Michaelis and Menten (13) for the hydrolysis of sucrose

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by invertase, has often been applied in studies on enzyme-catalyzed reactions. It yields a numerical value of the equilibrium constant for the dissociation of the postulated enzyme-substrate complex. Haldane (9) presents a compilation of the so-called Michaelis constants for a large number of reactions. His table shows, in general, constants of the order of magnitude of 10^{-2} for hydrolytic reactions and 10^{-6} for oxidative reactions, although exceptions to this general rule occur.

An analysis of the reaction of *Cypridina* luciferin and luciferase by Moelwyn-Hughes (14) did not give satisfactory agreement with the Michaelis-Menten theory of intermediate enzyme-substrate complex formation. Because the data used were from studies involving crude extracts of *Cypridina* luciferin and luciferase and for other reasons,[2] new measurements have been made with the more highly purified luciferin and luciferase now available. These new data show the effect upon the velocity of the luminescent reaction of varying the luciferin concentration over a range of 1:200. They have been analyzed in terms of the Michaelis-Menten theory and yield a value for the Michaelis constant which is not inconsistent with other values for enzyme-catalyzed systems.

Apparatus and Methods

Measurements of the luminescent reaction were made with a modification of the apparatus described by Anderson (2), which determines total emitted light. In this way very dim light production can be accurately measured, since the instantaneous intensity is not recorded but, rather, the amount of light produced during the interval from the start of the reaction to any particular time. When total light is plotted against time from initiation of the reaction, the slope of the first part of the resulting curve is directly proportional to the initial velocity.

The luciferase used was an extract of 5 g. of dried, powdered *Cypridina* organisms in 100 ml. of distilled water. This was filtered and subjected to prolonged dialysis, first against running tap water, and finally against several changes of distilled water.

[2] The data used by Moelwyn-Hughes were those of Harvey and Snell (11), who studied the effect of total luciferin concentration (luciferin plus oxyluciferin) upon the velocity constant of the reaction, using the crude aqueous extracts of luciferin and luciferase which were at that time available. For reasons unknown at present the velocity constant obtained under such conditions varies with substrate concentration as well as with the concentration of enzyme. It decreases as the substrate concentration is increased, and approaches a minimum value. This is not true for the reaction of the more highly purified *Cypridina* luciferin and luciferase now available, for which it has been shown (8) that the velocity constant is practically independent of luciferin concentration and varies only with the concentration of enzyme, as would be expected. However, Moelwyn-Hughes' use of velocity constants, rather than velocities, would appear to be unjustified on purely theoretical grounds.

This treatment removed much dialyzable material and also caused the precipitation of a considerable quantity of inactive protein. The final product from this dialysis, suitably diluted, served as the enzyme stock solution.

The luciferin, extracted also from dried, powdered *Cypridina* organisms, was carried through two cycles of purification by the method of Anderson (3). The degree of purity of the product from this procedure, while not actually known, is very high compared with that of luciferin in a crude, aqueous extract of the organisms.[3]

Since the hydrogen ion concentration and that of other ions greatly affect this luminescent reaction (2,4,6) the reaction mixture used in these experiments always consisted of equal volumes of $M/15$ KH_2PO_4 and $M/15$ Na_2HPO_4 and was 0.01 M for NaCl.

The luciferin obtained from Anderson's purification procedure is in hydrogen-saturated *n*-butyl alcohol solution, stored under an atmosphere of hydrogen. Preliminary experiments showed that this alcohol has an inhibitory effect on the activity of the enzyme, luciferase. It was, therefore, necessary to use some other solvent for the luciferin since the substrate concentrations were to be varied in the experiments by taking different volumes of luciferin stock solution. 0.1 N HCl was chosen as the solvent. Luciferin stock solution for a day's experiments was prepared as follows. Two ml. of the butanol-luciferin solution were transferred from the storage vessel to a small vial. By means of a vacuum desiccator, a liquid nitrogen freezing trap and a vacuum pump, the alcohol was removed from the solution, leaving a solid residue. This was dissolved in 5 ml. of 0.1 N HCl and the resulting solution put into a small test tube immersed in an ice water bath. This last precaution is necessary because luciferin undergoes spontaneous oxidation in the presence of dissolved oxygen, losing the property of giving light with luciferase, and this oxidation is greatly retarded at low temperatures.

For an individual luminescence measurement the desired volume of luciferin-HCl stock solution (0.02–2.00 ml.) was placed in the bottom of the reaction vessel. Next, enough 0.1 N HCl was added to make the total volume of acid present (including that added with the luciferin) 2.00 ml. This was done to keep the pH and chloride ion content constant in all experiments. Ten ml. of the reaction mixture were next added and the reaction vessel was placed in position in the light-measuring apparatus. Another 10 ml. volume of reaction mixture containing the luciferase was then run in from a fast-flowing pipette, starting the luminescent reaction. Total emitted light was recorded at intervals for as long a period as desired.

Experimental Results

Exploratory experiments with ordinary dilutions of the luciferase stock solution showed that a linear relationship apparently existed between luciferin concentration and velocity of the luminescent reaction, with no indication of a maximum velocity. Evidently a high con-

[3] In terms of quantity of light produced per unit dry weight of material, the luciferin used in the present work was about 1600 times as pure as that in the dry powder obtained by grinding the whole *Cypridina* organisms.

centration of substrate was required to saturate the enzyme. Rather than use higher concentrations of luciferin, which was prohibitive because of the limited supply of *Cypridina* available, the concentration of enzyme was greatly decreased. With a 1:600 dilution of the stock luciferase solution a typical velocity *vs.* substrate concentration relationship was obtained. Luminescence was necessarily very dim at the

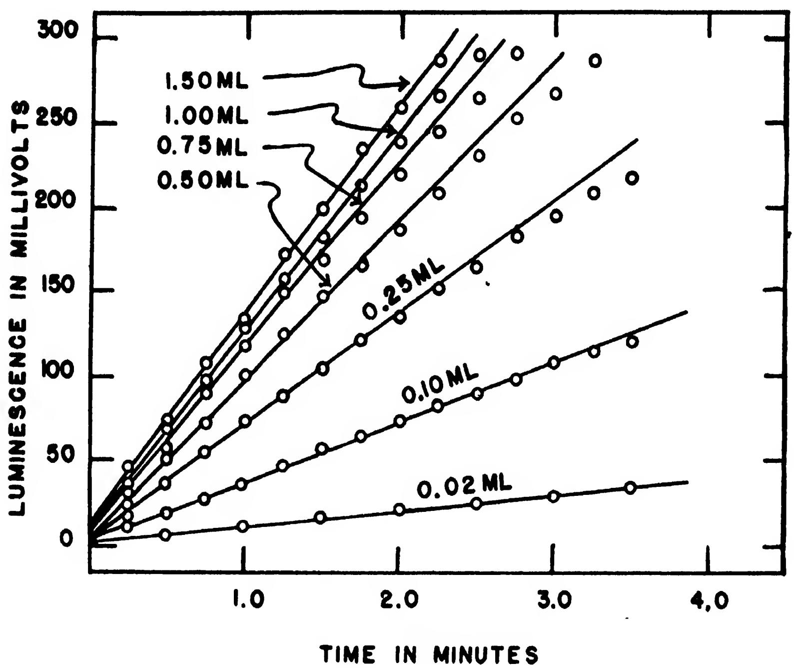

FIG. 1. Total light emitted during the first 2–3.5 min. after adding a constant, small amount of luciferase to various volumes of luciferin solution in a constant volume of reaction mixture. The straight lines represent the data during the early part of the reaction and their slopes are proportional to the initial velocity. The plotted values are from Table I.

lowest luciferin concentrations, but it could still be accurately measured with the light-integrating apparatus. Typical data are plotted in Fig. 1 and all numerical values are given in Table I. To avoid confusion in the figure, not all of the data have been plotted. Because of the very low luciferase concentration that was used, it was not practical to measure these luminescence reactions over their entire course but the initial

velocities can be determined very precisely from the slopes of the straight lines which fit the data in the early part of the reaction.[4]

Inspection of Fig. 1 shows that the slopes of the lines (proportional to initial velocity) increase asymptotically toward a maximum value as the luciferin concentration is increased. The relationship between initial

TABLE I

Luminescence, in Arbitrary Units (Millivolts), Obtained from a Constant Amount of Luciferase and Various Amounts of Luciferin Solution

These are the data as directly measured. They are plotted in Fig. 1, with the exception of those luminescence experiments involving 0.90, 1.80 and 2.00 ml. of luciferin solution.

Time	Total luminescence in millivolts for indicated volumes (in ml.) of luciferin solution; luciferase concentration constant									
	0.02	0.10	0.25	0.50	0.75	0.90	1.00	1.50	1.80	2.00
min.										
0.25	—	12	18	24	32	30	37	48	35	37
0.50	7	20	38	52	58	62	70	74	70	73
0.75	—	28	56	74	90	88	98	108	100	108
1.00	12	38	74	102	119	116	129	143	133	140
1.25	—	48	88	125	150	144	157	172	164	168
1.50	16	57	105	148	168	171	183	200	188	199
1.75	—	65	121	165	194	198	214	235	218	227
2.00	22	74	135	186	219	220	240	260	248	259
2.25	—	82	152	208	245	248	266	287	275	290
2.50	24	90	164	232	266	266	290	314	300	316
2.75	—	98	182	252	292	291	312	340	328	344
3.00	30	107	195	268	314	314	336	367	352	368
3.25	—	114	208	287	335	337	358	392	376	394
3.50	35	120	223	310	355	358	380	415	400	420

velocity of the luminescent reaction and luciferin concentration is, therefore, essentially the same as that encountered in general for enzyme-catalyzed processes.

[4] It will be observed that the straight lines of Fig. 1 do not pass exactly through the origin but slightly above it. This is probably because at the moment of mixing the luciferin and luciferase an initial flash of light occurs of complicated nature. This initial burst was first observed in the experiments of Amberson (1). It is small compared with the total light produced in the present experiments.

Discussion

Since the present data relating velocity of the *Cypridina* luminescent reaction to luciferin concentration appeared to conform to the Michaelis-Menten theory, an analysis was made in the following way.

The usual form of the Michaelis-Menten equation is:

$$v = \frac{V' (S)}{K_s + (S)},$$

where v = measured velocity of the reaction, V' = maximum velocity, (S) = substrate concentration and K_s = the dissociation constant of the enzyme-substrate complex (*i.e.*, the so-called Michaelis constant).

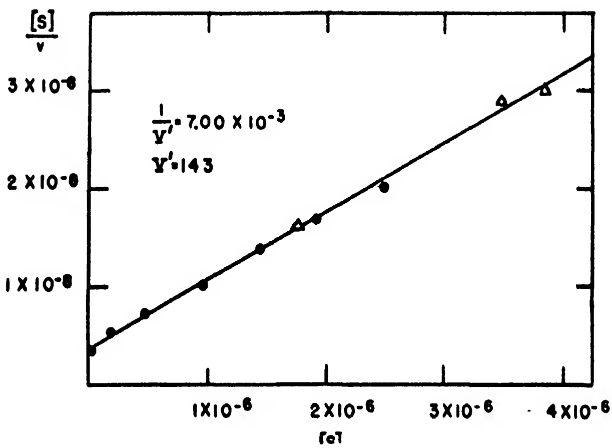

Fig. 2. The data of Fig. 1 plotted in terms of the equation, $(S)/v = ((S)/V') + (K_s/V')$, a linear modification of the Michaelis-Menten equation. Substrate concentration is expressed as molarity. The data are well fitted by a straight line, indicating that they obey the equation. The slope of this line is equal to the reciprocal of the maximum velocity, V', for the particular luciferase concentration used. The intercept is equal to the Michaelis constant divided by the maximum velocity. K_s, calculated from the intercept, is 5.95×10^{-7}.

The value of the maximum velocity must be known in order to calculate the Michaelis constant for the system but it was not possible, in the present case, to obtain this datum experimentally. Fortunately, this can be done graphically if the data are otherwise adequate. Lineweaver and Burk (12) have shown that if the reciprocal of the classical

Michaelis-Menten equation be multiplied through by (S), the following linear equation is obtained:

$$\frac{(S)}{v} = \frac{(S)}{V'} + \frac{K_s}{V'}$$

(all terms having the same meanings as before). If the equation is satisfied, a straight line should fit the data when $(S)/v$ is plotted against (S). The slope of this line is equal to the reciprocal of the maximum velocity, and the intercept gives the value of K_s/V'. Fig. 2 shows the data plotted in this form. The slope can be determined with considerable accuracy and yields a value for the maximum velocity of 143 millivolts/min., for the particular luciferase concentration used. Substrate concentration is expressed in terms of molarity[5] in Fig. 2, and K_s has a value[6] of 5.95×10^{-7}.

The results of another series of luminescence experiments, in which a higher luciferase concentration had been used, gave a value for K_s of 6.03×10^{-7}, not essentially different from that of the series with the lower enzyme concentration. Lack of space prohibits inclusion of the data from this second series of experiments but they are of the same quality as those shown in Figs. 1 and 2.

It is of course recognized that K_s may not represent the true dissociation constant of the luciferin-luciferase complex since, as Briggs

[5] The dry weight of luciferin used and its molecular weight must be known in order to express substrate concentration in terms of molarity. The molecular weight of *Cypridina* luciferin is not exactly known at present, although a determination of its combining weight was made by Chase (7), using a reaction between luciferin and ferricyanide. While an exact figure could not be obtained, his data indicated a combining weight somewhere between about 250 and 600. On the assumption that the combining weight as measured is equal to the molecular weight, and for purposes of calculating K_s in the present case, 500 was taken as the molecular weight of luciferin. By weighing the dry residue from a 20 ml. volume of n-butanol-luciferin solution it was found that each ml. contained 6×10^{-5} g. of solid. Since, in the present experiments luciferin stock solutions were made by redissolving the residue from 2.0 ml. of butanol-luciferin solution in 5.0 ml. of 0.1 N HCl, each ml. of the HCl-luciferin stock solution would contain 2.4×10^{-5} g. of solid. For purposes of calculation it was assumed that this solid is pure luciferin. These assumptions obviously affect the calculation of a precise value for K_s. However, they do not particularly affect the order of magnitude of the constant.

[6] If, in the present case, substrate concentrations are expressed as percentage concentration rather than in molarity, a value of 2.6×10^{-5} is obtained for K_s. Some of the constants in the literature have been calculated in this way for reactions where the molecular weight of the substrate is quite unknown.

and Haldane (5) pointed out, the theory of Michaelis and Menten assumes the velocities of formation and dissociation of the enzyme-substrate complex to be infinitely great compared to the velocity with which the end-products of the reaction are split off from the intermediate complex. The theoretical treatment, therefore, ignores these first two velocity constants and considers the third only. This assumption may not be justified in all cases. However, taking the value of K_s for the luminescent reaction at its face value, the luciferin-luciferase intermediate complex would appear to be similar in nature to those of oxidative enzyme systems in general, since such systems exhibit relatively low K_s values as compared with hydrolytic enzyme systems. There seems to be little doubt that the Michaelis-Menten theory applies in the case of the luminescent reaction of partially purified *Cypridina* luciferin and luciferase under the existing experimental conditions.

The very good description of these data by the Michaelis-Menten equation also indicates that one molecule of luciferin combines with one luciferase molecule in the formation of the enzyme-substrate complex. This conclusion was further substantiated by plotting the data in terms of an equation representing the reversible formation of an enzyme-substrate complex involving 2 molecules of luciferin for each molecule of luciferase instead of the 1:1 ratio. The equation has the form:

$$\frac{1}{v} = \frac{K_s}{V'} \cdot \frac{1}{(S)^2} + \frac{1}{V'}.$$

If it describes the data, a straight line should be obtained when $1/v$ is plotted against $1/(S)^2$. The data, plotted in this way, could not be fitted by a straight line. It can, therefore, be concluded with some certainty that the intermediate enzyme-substrate complex for this reaction involves not more than one molecule of luciferin for each molecule of luciferase.

Acknowledgment

It is a pleasure to acknowledge the capable technical assistance of Miss Betsy Brigham, who performed most of the experiments upon which this paper is based.

Summary

Enzyme-catalyzed oxidative bioluminescent reactions have the unique property that the intensity of the luminescence affords a direct, instantaneous measure of the velocity of the underlying process.

The luminescent reaction of *Cypridina* luciferin and luciferase, both partially purified, was measured under conditions where, with constant enzyme concentration, the substrate concentration was varied over a 200-fold range. The velocity of the reaction increases with increase of luciferin concentration and approaches a maximum value asymptotically.

The Michaelis constant, K_s, representing the dissociation of an enzyme-substrate complex, was calculated and found to be about 6×10^{-7}. This order of magnitude resembles those found for many oxidative enzyme systems.

The analysis of the data indicated that not more than one molecule of luciferin combines with one molecule of luciferase in forming the enzyme-substrate complex.

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Studies with Penicillinase in the Presence of Sulfonamides [1]

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Introduction

Recent studies indicated that penicillin and sulfonamides in combination may act synergistically *in vitro* and *in vivo* (2,4,8). To explain this synergistic action, it has been suggested (7) that penicillin reduces the number of bacteria to limits within which the sulfonamides become completely inhibitory.

An investigation of penicillin blood levels in normal adults after varying doses of penicillin given orally together with sulfonamides and alkalizing buffer salts, showed that the values were fairly high and quite uniform (1). While the buffer salts (sodium citrate and sodium lactate) accounted to a large extent for the good utilization of the ingested penicillin the question arose whether the sulfonamide provided additional protection, particularly against the destructive action of penicillinase.

Winnek (11) combined penicillin with phthalylsulfathiazole in rectal suppositories to prevent the growth of penicillinase-producing bacteria in the large bowel. From the penicillin blood levels obtained, it was concluded that the drug had been protected effectively.

While it had been reported (5) that sulfadiazine sodium was without any effect on penicillinase, our experiments indicated a definite interaction between sulfonamides and penicillinase.

Experimental

A weighed amount of penicillinase was dissolved in 25 ml. of distilled water, the sulfonamide was suspended, and the mixture heated to 37°C. Ten ml. of a penicillin solution pre-heated to 37°C. was added, and a sample taken immediately for penicillin assay. The mixture was kept at 37°C. and one or more samples withdrawn at

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specified intervals. All samples were diluted with water to a theoretical penicillin content of 1 unit/ml. and assayed by the agar cup method using *Staph. aureus* (12). Control experiments without sulfonamides were always made simultaneously.

The activity of the penicillinase (Takamine Laboratories) used in most of this study was determined for varying enzyme concentrations and two penicillin levels and for different times of exposure. Complete inactivation in 30 min. at 37°C. of 10,000 or 50,000 units of penicillin required about 57 and 300 mg., respectively, of the enzyme. When the time of exposure is increased, smaller amounts of penicillinase will effect complete destruction of penicillin.

TABLE I

Inactivation of Penicillin by Varying Amounts of Penicillinase without and with Addition of a Mixture of 500 mg. Each of Microcrystalline Sulfadiazine and Microcrystalline Sulfathiazole

Penicillinase	10,000 Units penicillin		Penicillinase	50,000 Units penicillin	
	After 30 min. incubation[a]			After 30 min. incubation[a]	
	Without sulfonamides	Sulfa mixture added		Without sulfonamides	Sulfa mixture added
mg	*zone of inhibition in mm.*		*mg.*	*zone of inhibition in mm.*	
0	7.5	7.4	0	7.5	7.7
25	7.2	7.7	100	7.5	7.8
50	4.0	7.6	150	7.5	7.6
60	0	7.3	200	3.8	6.3
80		7.2	300	0	5.6
100		3.8	400		0
150		0			

[a] The zone of inhibition before incubation is the same in all experiments, averaging 7.6 mm.

After the activity of the penicillinase had been established, the effect of sulfonamides was studied. In the first experiments, 500 mg. each of microcrystalline sulfadiazine and microcrystalline sulfathiazole were suspended in the reaction mixture. With an exposure of 30 min., and with both 10,000 and 50,000 units of penicillin, the sulfonamides provided a marked protection against penicillinase (Table I). The same protection was obtained when the amount of enzyme was kept constant and the exposure was varied (Table II). The figures also indicate that it makes no difference whether sulfadiazine or sulfathiazole, or a mixture of these two drugs, is added. Sulfonamides alone do not give any zone of inhibition in the high dilution in which the solutions are used in the cup test.

Since the zone of inhibition before incubation is the same in all experiments and equals that found before and after incubation without any penicillinase added, it can be concluded that the small amount of enzyme left after dilution for the cup test has no measurable effect on the penicillin determination.

TABLE II

Inactivation of Penicillin by Penicillinase in the Presence of Sulfonamides after Varying Times of Exposure at 37°C.[a]

Time of incubation	10,000 Units penicillin + 25 mg. penicillinase			
	Without sulfonamides	Sulfa mixture added[b]	Sulfathiazole added[c]	Sulfadiazine added[d]
min.	*zone of inhibition in mm.*			
0	7.2	7.3	7.4	7.2
30	6.2	7.2	6.5	6.8
60	4.1	6.8	5.8	6.3
90	1.9	6.3	5.5	5.9
120	0	5.6	5.0	5.4

[a] The zone of inhibition before incubation is the same in all experiments, averaging 7.6 mm.

[b] 500 mg. each of microcrystalline sulfadiazine and sulfathiazole.

[c] 1 g. of microcrystalline sulfathiazole.

[d] 1 g. of microcrystalline sulfadiazine.

The experimental data seemed at first to present a case of true enyzme inhibition by sulfonamides. The first indication that the results had to be explained by a different mechanism, however, was seen in the experiments with single sulfonamides (Table II). The protection by either sulfathiazole or sulfadiazine alone, or by a mixture of these two drugs, was practically the same, even though sulfathiazole is about 8 times more soluble in water at 37°C. than sulfadiazine. Furthermore, saturated solutions of one or mixtures of several sulfonamides did not provide any protection. However, all

TABLE III

Protection of Penicillin (10,000 Units) against Penicillinase by Varying Amounts of Microcrystalline Sulfathiazole

Time of exposure: 30 min. at 37°C.

Penicillinase	Without sulfonamide	Microcrystalline sulfathiazole added		
		1 g.	2 g.	3 g.
mg.	*zone of inhibition in mm.*			
25	7.8	7.4		
50	4.5	7.2	6.8	6.0
100	0	5.2	6.3	5.0
150		0	4.2	3.0
200			0	1.6
250				0

observed facts were in agreement with the assumption that the penicillinase was adsorbed by the suspended sulfonamide particles and thus removed from the solution.

To prove this assumption, the amount of microcrystalline sulfathiazole was changed and it was found (Table III) that larger quantities will provide full protection against increasing concentrations of penicillinase. Within the range of the experiments a direct relationship exists between the amounts of sulfathiazole and inactivated penicillinase. Since 1 g. of sulfathiazole is already far in excess of its solubility at 37°C. in the volume used, an increase to 2 or 3 g. will not change this situation. Thus, the protection against higher concentrations of penicillinase points to an adsorption of the enzyme by sulfathiazole particles.

A comparison of microcrystalline sulfathiazole (particle size about 5 μ) with regular sulfathiazole (50 to 100 μ) showed (Table IV) that the protective action of regular sulfathiazole is markedly less than that of the microcrystalline form. Since the only difference is in the particle size of sulfathiazole, the conclusion seems justified that the penicillinase is removed from the solution by physical adsorption.

TABLE IV

Protection of Penicillin against Penicillinase by Microcrystalline and Regular Sulfathiazole

10,000 units of penicillin and 2 g. of sulfathiazole.

Penicillinase	Microcrystalline sulfathiazole		Regular sulfathiazole	
	Before incubation	After 30 min. incubation	Before incubation	After 30 min. incubation
mg.	*zone of inhibition in mm.*		*zone of inhibition in mm.*	
50	7.5	6.3	7.6	5.8
60	7.7	6.3	7.5	5.0
70	7.6	5.8	7.5	5.3
80	7.3	6.3	7.4	3.9
90	7.1	5.0	7.3	0
100	6.9	4.1	6.8	0

The time required for the adsorption of penicillinase by the sulfonamides is fairly short. When a solution of the enzyme (50 mg.) is shaken continuously with 2 g. of the microcrystalline sulfa drug in the absence of penicillin, then centrifuged, and the supernatant tested for enzyme activity, the adsorption is found to be complete within 5 min. This experiment may also serve as a final proof for the adsorption theory, since a solution of penicillin alone is not affected, even after prolonged contact of several days with microcrystalline sulfonamides.

The foregoing experiments had all been made with a rather crude penicillinase preparation, and it seemed desirable to test the behavior of a purified form of this enzyme, such as penicillinase A-Schenley.[3] Our data indicate that, on a weight basis, this preparation is approximately 500 times more potent.

[3] We are indebted to Dr. C. E. Duchess, Schenley Laboratories, Inc., for a supply of Penicillinase A.

In the experiments, the enzyme solution (20 ml.) was shaken for 5 min. with the specified amount of microcrystalline sulfadiazine, then centrifuged, and 10 ml. of the clear supernatant used for the test as previously described. Controls without the sulfonamide were treated in an identical manner.

TABLE V

Protection of Penicillin (50,000 Units) against Purified Penicillinase by Microcrystalline Sulfadiazine

Time of exposure: 60 min. at 37°C.

Penicillinase	Without sulfadiazine	After treatment with microcrystalline sulfadiazine	
		1 g.	2 g.
mg.	*zone of inhibition in mm.*		
None	7.9	7.9	8.0
0.09	5.7	7.7	7.7
0.18	4.4	6.5	7.1
0.27	2.1	5.6	6.3
0.36	0	4.2	6.0
0.54		0	4.3

The results (Table V) show that there is no difference between the crude and the purified enzyme preparation as far as the adsorption of the penicillinase is concerned. It should be noted that the amount of sulfonamide required for complete adsorption is of the same order if the enzyme preparations are compared on the basis of their activity.

Preliminary experiments with microcrystalline sulfaguanidine indicate that the adsorptive power of this compound for penicillinase is much lower than that of either sulfadiazine or sulfathiazole.

Discussion

The experimental observations are of interest for two reasons. First, penicillin can be protected by sulfonamide suspensions against the destructive action of penicillinase. Whether or not such a protection is of practical importance in the oral administration of penicillin requires further investigation. According to Stewart and May (10), the amount of penicillinase found in the upper part of the human digestive tract is negligible. However, their conclusion is based only on indirect proof in a small number of experiments. In mice and rabbits, the penicillin blood levels may be prolonged when the drug is given orally together with substances which *in vitro* have an inhibiting effect on penicillinase (9).

The second factor concerns the observation that suspensions of sulfonamides may remove, at least temporarily, an enzyme from its solution by physical adsorption. Such adsorption is observed frequently and has been utilized in the purification of penicillinase (6). The same principle is also applied in therapeutics by using insoluble substances such as kaolin in the treatment of certain intestinal disorders (3). However the present observation is apparently the first example that a drug which is given orally for its systemic effect may also act, at least temporarily, by adsorbing enzymes or other high molecular compounds. It should be mentioned that sulfonamides are also frequently applied in the form of dusting powder or ointments to wounds and body surfaces. In these instances the possibility of interfering with normal biochemical processes is even greater, since the time of contact is much longer than in the case of oral administration.

Summary

The presence of sulfonamides (sulfadiazine and/or sulfathiazole) results in a marked protection of penicillin against penicillinase. This protection is due to an adsorption of the enzyme on the sulfonamide particles. The degree of protection depends upon the amount of suspended sulfonamides and their particle size.

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Antibiotin Effect of Homologs of Biotin and Biotin Sulfone [1,2]

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Introduction

Hofmann, Chen, Bridgwater and Axelrod (1) have described the biological activity of a number of homologs of O-heterobiotin (oxybiotin), including the bis-nor, nor, homo and bis-homo compounds. None of these were active antibiotins for either *S. cerevisiae* or *L. arabinosus*. On the other hand, the nor, the bis-homo and the homo compounds showed anti-O-heterobiotin activity for *S. cerevisiae*, while for *L. arabinosus* only the last compound was active. Since Goldberg *et al.* (2) have synthesized comparable homologs in the *biotin* series, it became possible to extend the studies to homologs of biotin and biotin sulfone and to compare, in a general fashion, the activities of the homologs of biotin and O-heterobiotin.

Experimental

Methods

The antibiotin activity of the homologs was investigated with *Saccharomyces cerevisiae 139*, *Lactobacillus casei* ϵ and *Lactobacillus arabinosus 17-5*. The procedures used with *S. cerevisiae* and *L. casei* have been reported elsewhere (2,3,4), with the present difference that the yeast assays were carried out in flasks. *L. arabinosus* was grown in the medium of Wright and Skeggs (5) and, in most of the experiments, incubated at 30°C. for periods of 20 or 67 hr. However, as it was found that greater growth was obtained in a shorter period at 37.5°C., some of the later experiments on the reversal of inhibition by biotin and the effect of the homologs in the presence of Tween 80 were read after 18 hr. at the higher temperature. The growth was measured turbidimetrically.

The antibiotin activity expressed as the Molar Inhibition Ratio (M.I.R.) (6) was

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determined in the following ranges: *L. casei* 0.1–0.2 mγ, *S. cerevisiae* 0.1–0.2 mγ, and *L. arabinosus* 0.5–1.0 mγ; anti-O-heterobiotin activity was determined in the ranges: *L. casei* 0.6–1.0 mγ, *L. arabinosus* 1.0–2.0 mγ, and *S. cerevisiae* 0.5–1.0 mγ.

The common denominator in these various ranges for the estimation of M. I. R. has been the choice of comparable *linear* portions of the curves of response (*cf.* (4) for typical assay curves). The reproducibility of these determinations of M. I. R. can vary with many factors, including the slope of the curve, time of incubation, concentration of inoculum, *etc.*, with the consequence that we have not always been able to reproduce values within the limits of ± 20% claimed by Dittmer and du Vigneaud (7). However, the M. I. R. reported here and elsewhere for various antibiotins cover so wide a range, that variations up to 100% are not of great consequence.

Results

Activity for S. cerevisiae (Table I)

Either decreasing or increasing the biotin side chain by one methylene group gives compounds (nor- and homobiotin, respectively) which are potent antagonists of biotin and O-heterobiotin. Further increase in the side chain length (bis-homo- and tris-homobiotin) progressively diminishes the antibiotin activity.

On the other hand, among the homologs of biotin sulfone, the nor-compound is by far the weakest antimetabolite while the remaining homologs show approximately the same activity. These sulfone homologs are, on the whole, less effective in antagonizing the growth of yeast than the corresponding biotin homologs.

When the M.I.R. is determined by the depression of growth from 0.2 to 0.1 mγ of biotin, the homologs of biotin and biotin sulfone, with the exception of nor- and homobiotin, are 11–30 times more effective against O-heterobiotin than against biotin. On the other hand, the similarity in antibiotin and anti-O-heterobiotin activity of nor- and homobiotin is surprising, both with respect to the general pattern of the present study and the work of others (1,4,7,11). However, when the M.I.R. is calculated from total inhibition (Table IV), norbiotin and homobiotin are many times more effective against O-heterobiotin than against biotin.

Activity for the Lactobacilli (Table I)

Homobiotin and its sulfone are the most potent inhibitors, the M.I.R. against biotin being 130 and 460, respectively, for *L. casei*, and 4000 and 1200, respectively, for *L. arabinosus*. Tris-homobiotin sulfone (M.I.R. 6200) is also a potent antibiotin for the latter organism.

TABLE I

Antibiotin and Anti-O-Heterobiotin Effect of Homologs of Biotin and Biotin Sulfone for Microorganisms

Homologs	n[a]	Molar inhibition ratio[b]								
		S. cerevisiae			*L. casei*			*L. arabinosus*		
		Antibiotin	Anti-O-H-biotin	Antibiotin / Anti-O-H-biotin	Antibiotin	Anti-O-H-biotin	Antibiotin / Anti-O-H-biotin	Antibiotin	Anti-O-H-biotin	Antibiotin / Anti-O-H-biotin
DL-Norbiotin	3	900	700	1.3	24,000	1,300	18	>200,000	17,000	>12
DL-Homobiotin	5	500	700	0.7	130	8	16	4,000	100	40
DL-Bis-homobiotin	6	20,000	1,300	15	7,200	160	45	>200,000	7,300	>27
DL-Tris-homobiotin	7	50,000	1,800	28	3,000	45	67	63,000	3,700	17
DL-Norbiotin sulfone	3	210,000	16,000	13	60,000	3,600	17	>200,000	3,000	>67
DL-Homobiotin sulfone	5	40,000	3,500	11	460	18	26	1,200	100	12
DL-Bis-homobiotin sulfone	6	60,000	2,000	30	8,000	600	13	13,000	2,200	6
DL-Tris-homobiotin sulfone	7	50,000	2,300	22	6,000	240	25	6,200	850	7

[a] n is the number of methylene units in the side chain; $n = 4$ in biotin and biotin sulfone.

[b] Determined for *S. cerevisiae* after 16 hr. at 30°C., for *L. casei* after 3 days at 37.5°C., and for *L. arabinosus* after 20 hr. at 30°C.

d-Homobiotin was found to be twice as active as *dl*-homobiotin for *L. arabinosus*, demonstrating that only one of the enantiomorphs is active.

For *L. casei*, the biotin homologs are 16–67 times more effective in inhibiting growth when O-heterobiotin is the growth factor. The ratio for the sulfone analogs is somewhat more constant, varying from 13 to 26. Among the biotin homologs, the homo- and tris-homo-compounds are very potent, while among the homologs of biotin sulfone, only the homo-compound shows a marked anti-O-heterobiotin effect. *The molar*

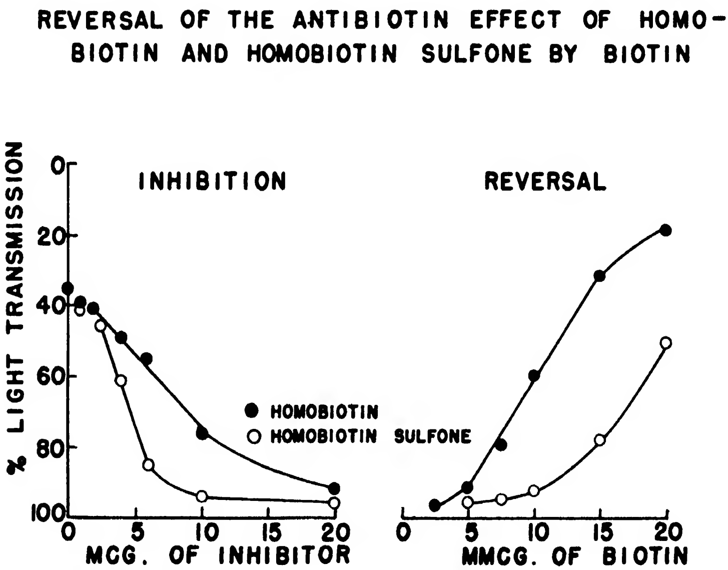

FIG. 1. The incubation period for the test organism, *L. arabinosus*, was 18 hr. at 37.5°C. The effect of each inhibitor was measured in the presence of 1.0 mγ of biotin per tube while the effect of biotin in reversing the antibiotin activity was measured in the presence of 100 γ of inhibitor.

inhibition ratios of homobiotin and homobiotin sulfone against O-heterobiotin (8 and 18, respectively) are the strongest antivitamin effects yet recorded in the biotin literature.

For *L. arabinosus* the homologs are from 6 to 67 times more effective in antagonizing growth when O-heterobiotin is the growth factor. Homobiotin (M.I.R. 100), homobiotin sulfone (M.I.R. 100), and tris-homobiotin sulfone (M.I.R.850) are the most potent anti-O-heterobiotins. When the incubation period is extended from 20 to 67 hr.,

there is a marked decrease in antibiotin effect (compare the *L. arabinosus data in Tables I and III*).

The biotin homologs are, in general, more effective than the biotin sulfone homologs against *L. casei*, while the reverse is true for *L. arabinosus*.

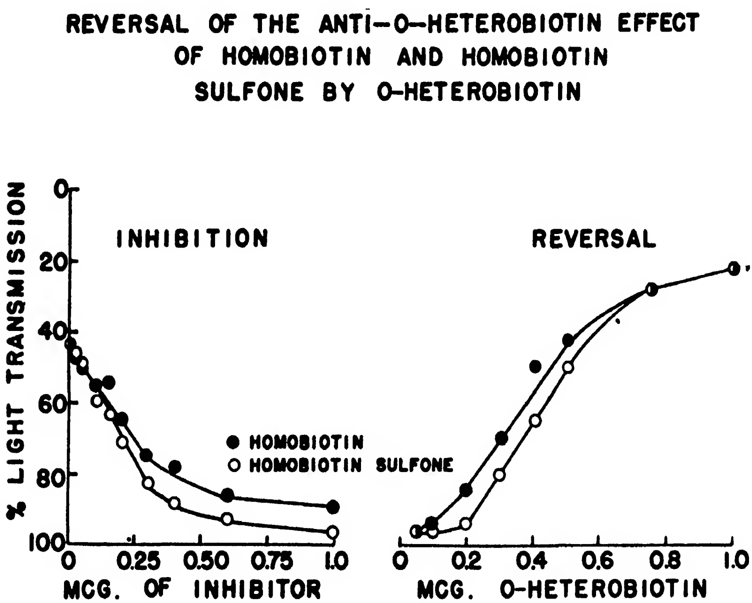

FIG. 2. The incubation period for the test organism, *L. arabinosus*, was 18 hr. at 37.5°C. The effect of each inhibitor was determined in the presence of 2.0 mγ of O-heterobiotin per tube, and the effect of O-heterobiotin in reversing the anti-O-heterobiotin activity of homobiotin and homobiotin sulfone was determined in the presence of 100 γ of inhibitor.

Reversal of Inhibition by Biotin and O-Heterobiotin

The effect of increasing amounts of biotin in nullifying the antibiotin effect of homobiotin, tris-homobiotin, homobiotin sulfone and tris-homobiotin sulfone, was determined with *L. arabinosus* (18 hr. at 37.5 C.). For the biotin homologs, about 5.0 and 0.5 mγ of biotin were required to initiate growth in the presence of 100 γ of homobiotin and tris-homobiotin, respectively, while for the biotin sulfone homologs about 15 and 1.0 mγ of biotin initiated growth in the presence of 100 γ of homobiotin sulfone and tris-homobiotin, respectively. The reversal

of inhibition by biotin, as well as the antibiotin effect of homobiotin and homobiotin sulfone, are illustrated in Fig. 1.

O-heterobiotin also reverses the effect of these antagonists, as is illustrated in Fig. 2. Similarly, for *S. cerevisiae*, the antibiotin effect of the homologs is reversed by biotin and O-heterobiotin.

TABLE II

Estimates of Molar Inhibition Ratio for L. arabinosus from Partial and Total Inhibition[a]

Compound	Molar inhibition ratio[b]					
	Antibiotin			Anti-O-heterobiotin		
	Partial inhibition	Total[c] inhibition	Total/Partial	Partial inhibition	Total[d] inhibition	Total/Partial
DL-Homobiotin	12,000	20,000	2	150	500	3
DL-Homobiotin sulfone	5,500	17,000	3	120	240	2

[a] Total inhibition ratios represent the amount of inhibitor required to prevent growth in the presence of either 1.0 mγ of biotin or 2.0 mγ of O-heterobiotin.
[b] Determined after 18 hr. at 37.5°C.
[c] Calculated from "inhibition" curve of Fig. 1.
[d] Calculated from "inhibition" curve of Fig. 2.

Comparison of Estimates of M.I.R. from Partial and Total Inhibition (Table II)

A comparison of the M.I.R. calculated in the usual fashion for *L. arabinosus* and the M.I.R. calculated at total inhibition of 1.0 mγ of biotin or 2.0 mγ of O-heterobiotin, shows that the partial inhibition values average approximately one-third of the total inhibition results, *i.e.*, values obtained from partial inhibition indicate that the compounds are 3 times more effective than values obtained from total inhibition. This is to be expected, since calculations of this nature from a sigmoidal curve would vary with the changing slopes of the curve. Our original choice of a method which measures partial inhibition is predicated largely upon use of a major part of the curve which is essentially linear (*cf.* "Methods").

As Hofmann *et al.* (1) used a comparable method for determining M.I.R. of O-heterobiotin homologs at total inhibition, this factor of 3 provides a basis for comparing their results with the present data, with the following results.

Comparison with O-Heterobiotin Homologs

In Table III the activity of the biotin homologs and the corresponding O-heterobiotin homologs for *L. arabinosus* is compared (incubation period: 3 days at 30 C.). As the activity of the O-heterobiotin homologs (nor-, homo- and bis-homooxybiotin) was reported by Hofmann *et al.* (1) on the basis of total inhibition, their results have been divided by the factor of 3 discussed in the preceding section. Among the O-heterobiotin homologs, the most active, homooxybiotin, is effective only against O-heterobiotin. On the other hand, the corresponding biotin homolog, homobiotin, not only is many times more effective against O-heterobiotin, but also antagonizes biotin. Bis-homobiotin is also an effective antagonist of O-heterobiotin.

TABLE III

Comparative Antibiotin and Anti-O-Heterobiotin Effect of Homologs of Biotin and O-Heterobiotin for L. arabinosus

Biotin homolog	Molar inhibition ratio				
	Present paper[a]		Hofmann *et al.* (1)[b]		
	Antibiotin	Anti-O-heterobiotin	O-Heterobiotin homolog	Antibiotin	Anti-O-heterobiotin
Nor-	>200,000	>100,000	Nor-	>167,000	>167,000
Homo-	29,000	940	Homo-	>167,000	75,000
Bis-homo-	>200,000	55,000	Bis-homo-	>167,000	>167,000

[a] Determined after 67 hr. at 30°C.

[b] These results have been divided by 3 for comparative purposes (*cf.* Table II and text).

For *S. cerevisiae* (Table IV), homooxybiotin shows a weak antibiotin activity, while the nor- and bis-homo- homologs are inactive. On the other hand, the corresponding biotin homologs are effective biotin antagonists. Against O-heterobiotin, both series of homologs are active, but again the biotin homologs are more effective antagonists.

As mentioned previously, the results in Table IV differ from those presented in Table I, particularly with respect to norbiotin and homobiotin. The data in Table IV were obtained by determining the amount of inhibitor required to antagonize completely 0.2 mγ of biotin or 1.0 mγ of O-heterobiotin, this method being comparable to the type of

TABLE IV

Comparative Antibiotin and Anti-O-Heterobiotin Effect of Homologs of Biotin and O-Heterobiotin for S. cerevisiae

Biotin homolog	Molar inhibition ratio				
	Present paper[a]		Hofmann *et al.* (1) and Axelrod *et al.* (13)		
	Antibiotin	Anti-O-hetero-biotin	O-Heterobiotin homolog	Antibiotin	Anti-O-hetero-biotin
Nor-	25,000	1,000	Nor-	>500,000	143,000
Homo-	25,000	1,000	Homo-	445,000	7,400
Bis-homo-	50,000	1,800	Bis-homo-	>500,000	30,000

[a] The molar inhibition ratios represent the amount of inhibitor required to prevent growth in the presence of 0.20 mγ of biotin or 1.0 mγ of O-heterobiotin.

measurement used by Hofmann *et al.* (1). A major change in antibiotin activity is found only in norbiotin and homobiotin, while bis-homobiotin, tris-homobiotin and the homologs of biotin sulfone show little change in antibiotin effect. The anti-O-heterobiotin activity of the homologs is not markedly affected by the change in method of determination of M.I.R.

Effect of Homologs in the Presence of Tween 80

Williams, Broquist and Snell (8) demonstrated that Tween 80 supports near-maximal growth of several microorganisms on a semi-synthetic medium in the absence of biotin. Potter and Elvehjem (9) later showed that a combination of aspartic acid and oleic acid would replace biotin almost completely for growth of *L. arabinosus*. These observations suggested that one function of biotin is concerned with the synthesis of oleic acid (8,9). If oleic acid is the product of an enzyme system catalyzed by biotin, then compounds antagonistic to biotin should have no effect on oleic acid. In Fig. 3 this is shown to be the case when Tween 80 replaces biotin. As the same result is obtained with *d*-homobiotin, tris-homobiotin, homobiotin sulfone, and tris-homobiotin sulfone, only the non-effect of *d*-homobiotin is shown. It was found that 750 γ of Tween 80 per tube produce in 18 hr. at 37.5 C. about the same growth response of *L. arabinosus* as 1 mγ of biotin. The addition of 400 γ of *d*-homobiotin has no effect on growth with oleic

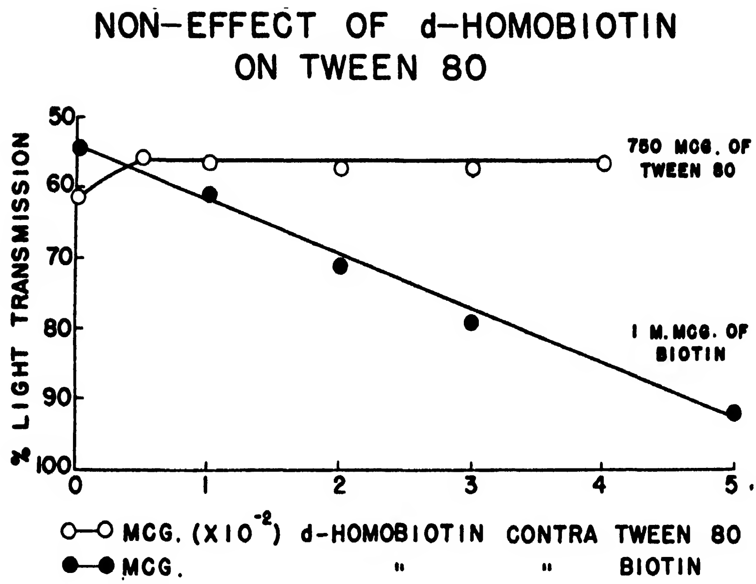

FIG. 3. The incubation period for the test organism, *L. arabinosus*, was 18 hr. at 37.5°C.

acid, while 5γ of inhibitor are sufficient to prevent growth with biotin. This specific effect of the homologs is similar to that of γ-(3,4-ureylene-cyclohexyl)-butyric acid (10). The failure of these inhibitors to prevent growth with oleic acid provides further evidence for the hypothesis that one function of biotin concerns the synthesis of oleic acid.

Discussion

The high activity of norbiotin in relation to homobiotin for *S. cerevisiae* can be contrasted with the results obtained by Dittmer and du Vigneaud (7) with imidazolidone aliphatic acids, and by Hofmann *et al.* (1) with homologs of oxybiotin. The former found that shortening the aliphatic side chain of imidazolidonecaproic acid by one methylene group reduced the antibiotin effect to a much greater extent than when the side chain was lengthened by one or two methylene groups. The latter found that nor-oxybiotin was a less effective O-heterobiotin antagonist than either homooxybiotin or bis-homooxybiotin. On the other hand, the progressive decrease in activity shown in Table I as the side chain is lengthened, is in general agreement with the work of Dittmer and du Vigneaud (7). The markedly decreased antibiotin

activity of norbiotin and homobiotin when the M.I.R. is calculated from total inhibition is surprising in view of the slight change in antibiotin activity of bis-homobiotin, tris-homobiotin and the homologs of biotin sulfone, as well as the slight changes in anti-O-heterobiotin activity found for all of the compounds. These results indicate that, although nor-biotin and homobiotin are effective biotin antagonists for the partial antagonism of growth, they are much less effective in antagonizing the growth of the yeast in the presence of the small amounts of biotin required to initiate growth. The observation that for both *L. casei* and *L. arabinosus* the antibiotin and anti-O-heterobiotin effect of the homologs higher than biotin is greater than the corresponding effect of the homolog lower than biotin, is in agreement with the results of Hofmann *et al.* (1) and Dittmer and du Vigneaud (7). However, in contrast to the results with *S. cerevisiae*, there is no progressive decrease in antagonism as the side chain is lengthened, as was reported by the latter group for imidazolidone aliphatic acids.

The greater antimetabolite effect of the homologs against O-heterobiotin than biotin is in agreement with previous observations on the comparative effect of desthiobiotin (4), of various homologs of O-heterobiotin (1), of biotin sulfone (11) and of desthiobiotin analogs (12). The antibiotin effect of *dl*-homobiotin (M.I.R.-4000) is much greater than that previously reported (M.I.R. 42,000) (2). However, the result reported initially was a range finder and, therefore, was indicative only of order of magnitude.

Summary

Norbiotin, homobiotin, bis-homobiotin, tris-homobiotin and the corresponding homologs of biotin sulfone are potent antibiotins for *L. casei* and, with the exception of the nor homologs and bis-homobiotin, for *L. arabinosus*. For *S. cerevisiae* all homologs but norbiotin sulfone are potent biotin antagonists.

When O-heterobiotin is the growth factor, the homologs are more effective against the lactobacilli and the yeast. The biotin homologs are generally more active against *S. cerevisiae* and *L. casei* than the biotin sulfone homologs, while the latter are more effective against *L. arabinosus*. The inhibition has been shown to be competitive in nature for *L. arabinosus* and *S. cerevisiae*.

The antibiotin activity of norbiotin and homobiotin against *S.*

cerevisiae is much greater when determined from partial than from total inhibition. This difference was not found for bis- and tris-homobiotin or for any of the sulfones tested. In the case of O-heterobiotin, the method of determination did not affect the M.I.R. of any of the compounds.

When oleic acid is the growth factor, homobiotin, tris-homobiotin, homobiotin sulfone, and tris-homobiotin sulfone have no effect on *L. arabinosus*.

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Biochemical Studies on Chloramphenicol (Chloromycetin).[1] I. Colorimetric Methods for the Determination of Chloramphenicol and Related Nitro Compounds [2]

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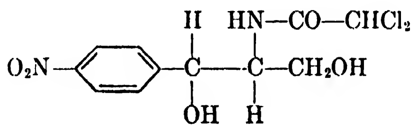
Introduction

Chloramphenicol (Chloromycetin[1]) is a new antibiotic which has proven to be highly effective against certain Rickettsiae and gram negative bacteria (1). It is an aromatic nitro compound which was isolated and partly characterized by Bartz (2). The complete structure has been established by Rebstock, Crooks, Controulis and Bartz (3), and it has been synthesized by several methods (4,5). Chloramphenicol is D(−)*threo*-2-dichloracetamido-1-*p*-nitrophenyl-1,3-propanediol, having the following configuration (3):



The analytical methods described in this paper involve the quantitative reduction of the aromatic nitro group to form a primary amine, which is then determined by the standard Bratton-Marshall diazo procedure (6). Aryl amines, which may be present before reduction, are accounted for by running a parallel determination in which the reduction step is omitted.

The chemical method is not specific for the active antibiotic, since inactive degradation products of chloramphenicol which still retain the nitro group are also included in the determination. Reliable estimates

[1] Parke, Davis and Co. trade mark for chloramphenicol.

[2] Reported in part at the Second National Symposium on Recent Advances in Antibiotic Research, Washington, D. C., April 11, 1949.

of active chloramphenicol are best made by microbiological assay procedures (1,7), and the differences between the chemical and microbiological assay figures thus represent inactive degradation products, including the glucuronide and the free base of chloramphenicol (8). The colorimetric method can be made more specific for the active antibiotic by using the solvent extraction procedure described in this paper.

A number of reducing agents were investigated for quantitative reduction of the nitro group in chloramphenicol, including titanous chloride, zinc, tin, Raney nickel, and electrolytic reduction. Of these, the first two agents were found to be most satisfactory. The titanous chloride reduction method was used extensively for biochemical investigations, and is preferred to the zinc method because no heating period is required for reduction and less interference is encountered from normal constituents of tissue. The zinc reduction method is included here because of its simplicity, but it is satisfactory only in the absence of interfering substances.

Experimental

Reagents

1. Zinc Dust. Reagent grade.

2. Titanous Chloride. An aqueous solution is made as described by English (9) and stored under hydrogen. Sixty ml. of stock 15% $TiCl_3$ is added to 300 ml. of concentrated HCl, heated to boiling and cooled under H_2 or CO_2. It is then poured into 3.6 l. of previously boiled and cooled distilled water in the storage bottle, air is displaced with H_2 and the contents thoroughly mixed. A convenient storage apparatus and burette is described by Knecht and Hibbert (10). The final solution should be a clear amethyst color, with no evident turbidity or precipitate.

3. Sodium Hydroxide. 1 *N* solution.

4. Hydrochloric Acid. 0.25 *N* and 0.5 *N* solutions.

5. Sodium Nitrite. 0.1% aqueous solution, prepared fresh daily.

6. Ammonium Sulfamate. 0.5% aqueous solution.

7. Bratton-Marshall Coupling Reagent. 0.1% aqueous solution of N-(1-naphthyl) ethylenediamine dihydrochloride. This should be stored in a brown bottle and kept in the refrigerator when not in use.

Procedure

Samples are diluted to contain no more than 120 γ of chloramphenicol/ml. Where appreciable amounts of protein are present, the samples are first deproteinized with 3% trichloroacetic acid for the titanium reduction method, or with $Zn(OH)_2$ if the zinc reduction procedure is to be used.

Reduction of the nitro group of chloramphenicol with titanous chloride proceeds rapidly and smoothly at room temperature. Excess titanium salts are precipitated as

the hydrated oxides in alkaline solution and removed by centrifugation to prevent interference with subsequent diazotization. An aliquot of the solution is then acidified and color is developed as described in the following section. A parallel determination is also run for aryl amines which may be present before reduction to correct for the color produced by these compounds.

One ml. of sample containing up to 120 γ of chloramphenicol is pipetted in each of two 15 $\times$ 125 mm. test tubes (A and B). To tube A is added 2 ml. of water, and 1 ml. of the titanous reagent is run in directly from the burette which is an integral part of the storage apparatus. The contents of the tube are immediately mixed by shaking. One ml. of 1 N NaOH is then added to precipitate the titanuim salts. The final pH should be approximately 9.0 to insure complete precipitation. With acid filtrates, the strength of the NaOH solution should be increased so that 1 ml. produces a final pH of 9.0. After formation of the precipitate, tube A is centrifuged at 1500 R.P.M. for a few min. to pack down the precipitate.

Four ml. of water is added to tube B, which serves as the aryl amine control. Finally, 1 ml. portions of the supernatant solutions from tubes A and B are transferred by pipette to two optical cuvettes (A and B), each containing 4 ml. of 0.25 N HCl. Color development is carried out as described in the next section. Where low concentrations of chloramphenicol are encountered, larger volumes of sample may be taken for reduction and for color development.

In the zinc reduction procedure, samples are diluted to contain less than 120 γ of chloramphenicol. One ml. of sample is pipetted into each of two 15 $\times$ 125 mm. test tubes (A and B) marked at the 5 ml. level. Four ml. of 0.5 N HCl is added to each tube, and approximately 50 mg. of zinc dust is added to tube A only. Both tubes are then heated in flowing steam or in a boiling water bath for exactly 1 hr. After cooling rapidly to room temperature, volumes are readjusted to the 5 ml. mark by the addition of water, ignoring the slight volume correction due to the presence of zinc ($<$0.01 ml.). Both samples are mixed thoroughly, the zinc is allowed to settle, and 1 ml. portions of the clear supernatant solution are transferred by pipette to 18 $\times$ 150 mm. optical cuvettes containing 4 ml. of 0.25 N HCl. These are then handled as described in the next section.

Development of Color

Following reduction by either the titanium or zinc procedures, samples are handled in the same manner thereafter for development of color. Prior to the diazotization procedure, if any appreciable amount of extraneous color is present in the cuvettes the optical densities should be read at 555 mμ in a Coleman Junior Spectrophotometer against a reagent blank. These readings are designated A_1 and B_1 for tubes A and B. Where the solutions are colorless, or nearly so, the A_1 and B_1 readings may be omitted.

To each cuvette is added 0.5 ml. of the $NaNO_2$ reagent, and the contents are mixed and allowed to stand for exactly 5 min. Then 0.5 ml. of the sulfamate reagent is added to decompose excess HNO_2. After 3 min. standing, 0.5 ml. of the Bratton-Marshall

reagent is added, and the contents of the tubes thoroughly shaken. All tubes are then placed in a water bath at 38°C. and left for exactly 1 hr. At the end of this time the tubes are removed, wiped dry with a clean towel, and optical densities are read against a reagent blank at 555 mμ (readings A_2 and B_2).

Calculation of Results

The optical density due to reducible nitro compounds is calculated from the colorimeter readings in the following manner:

$$\textit{Corrected Optical Density} = (A_2 - 0.77A_1) - (B_2 - 0.77B_1).$$

This equation corrects for any interfering color in the original sample (readings A_1 and B_1), and for the presence of aryl amines in the original sample (reading B_2). The factor 0.77 represents a correction for decrease in the optical density of the original solution due to dilution with the diazo reagents.

Corrected optical densities are converted to concentrations of chloramphenicol by reference to a standard curve prepared in the same manner as the unknowns, using standard solutions containing known concentrations of the drug. A direct relation between optical density and chloramphenicol concentration is found to exist. Final results are expressed in terms of "chloramphenicol-equivalents," since the inactive metabolic products containing the nitro group are not differentiated from chloramphenicol by this procedure. Analyses are run in duplicate on each sample, and at least one standard is included with each set of unknowns to check on the uniformity of test conditions.

In replicate analyses carried out with the zinc and titanous chloride procedures, variations of $\pm$ 4% from the mean were observed. After the addition of known amounts of chloramphenicol to plasma, recoveries of 92 to 98% of corresponding aqueous solutions are generally obtained. Recoveries of 90% were obtained with whole blood in dilutions of 1:40. Tissue homogenates gave recoveries of 90 to 98% in dilutions of 1:50, while lesser dilutions gave unsatisfactory results.

Conditions for Color Development

Optimum conditions for diazotization were established by studying maximum color formation with different concentrations of reagent, time of reaction and acidity. The final color was found to have maximum light absorption at 555 mμ, which is somewhat higher than found with simpler aryl amines (535 mμ–545 mμ). The same peak light absorption was found with zinc- and titanium-reduced samples, and the metabolic products of chloramphenicol showed similar absorption spectra.

The slow rate of coupling with the Bratton-Marshall reagent under the conditions of this procedure is evident by the slow increase in optical density on standing. Data presented in Table I show that the

coupling rate follows the pattern of a monomolecular reaction, and is accelerated at higher temperatures. Although complete color development is not attained in 1 hr. at 38°C., reproducible colorimetric readings can be made if standard solutions are also read in 1 hr. The coupling rate of the zinc-reduced samples appears to be somewhat greater than with the titanium-reduced samples, possibly due to splitting of the side chain on acid hydrolysis with the formation of a different reduction product.

TABLE I

Rate of Coupling of Reduced Chloramphenicol

Solutions containing 20 γ of titanium-reduced chloramphenicol were diazotized, sulfamate and coupling reagent added, and optical densities measured at 555 mμ after different periods of standing at 25°C. and 38°C. Readings were made against a reagent blank with a Coleman Junior Spectrophotometer. The rate constants are calculated from the equation $Kt = \log$ (*100/per cent remaining uncoupled*), where the greatest observed optical density is taken as 100% coupling, and t represents the time of standing in minutes.

Time	25°C.			38°C.		
	Optical density	Per cent coupled	K	Optical density	Per cent coupled	K
min.						
10	0.102	17	0.008	0.169	29	0.015
20	0.170	28	0.007	0.272	47	0.014
30	0.226	37	0.007	0.344	59	0.013
60	0.354	58	0.006	0.475	82	0.013
120	0.498	82	0.006	0.561	97	0.013
180	0.573	95	0.007	0.581	(100)	—
245	0.606	(100)	—	0.581	(100)	—

The rate of coupling is markedly influenced by pH, with the greatest rate observed at pH 4.5. However, at this pH a large part of the coupled compound exists as a yellow-colored base and the final solution must be further acidified to obtain maximum absorption at 555 mμ. This may be used as the basis for a rapid analytical procedure in which coupling is carried out at pH 4.5 for 20 min. at room temperature, and the solution is then acidified prior to reading the color intensity. The coupled compound appears to be fairly stable in acid and weakly alkaline solutions, but the diazonium salt prior to coupling is stable only in strongly acidic solutions. For this reason we prefer to conduct

the coupling reaction in strong acid, instead of using the rapid procedure at pH 4.5.

Establishment of Conditions for Reduction

Conditions for the reduction of chloramphenicol were established by varying time of heating, acid concentration and amount of reducing agent. The use of 0.25 *N* HCl and approximately 50 mg. zinc dust was found to give practically complete reduction in 1 hr. at 100°C. With titanous chloride, reduction was found to occur almost immediately at room temperature in acid, alkaline and neutral solutions. Six equivalents of titanous chloride are required for the reduction of one equivalent nitro group (10). However, a large excess of reagent is needed for complete reduction under the conditions of this test because no attempt is made to avoid atmospheric oxygen, and other reducible substances may be present.

The removal of titanium salts is accomplished by precipitation of the insoluble hydrated oxides in the presence of alkali.[3] This step is essential, because the titanous reagent would otherwise interfere with diazotization, reducing the HNO_2 and diazonium salts. Precipitation appears to be practically complete at pH 9, but a large excess of alkali should be avoided.[4] Aliquots of the clear supernatant solution should be removed for analysis immediately after centrifuging, because the titanous oxide is sufficiently reactive to decompose water slowly with the evolution of hydrogen gas (10), causing the precipitate to rise to the surface.

Solvent Extraction Procedure for Chloramphenicol

The colorimetric procedure described in this paper does not distinguish between active chloramphenicol and its inactive degradation products which retain the aromatic nitro group. The urinary excretion of these inactive metabolic products is often 10-fold greater than that of active antibiotic (11). Consequently, an extraction procedure was developed to increase the specificity of the colorimetric method for chloramphenicol.

The following method was used for analysis of urine samples collected from dogs which had been given chloramphenicol by mouth. One ml. of urine was pipetted into glass-stoppered tubes containing 6 ml. of ethyl acetate and 2 ml. of 0.2 *M* phosphate buffer at pH 6.0.[5] The mixtures were shaken in a mechanical shaker for 10 min. The tubes were then centrifuged and the aqueous layers removed by aspiration and discarded. The organic layer was washed twice by shaking with equal volumes of pH 6.0 buffer previously saturated with ethyl acetate. After separation of the two phases, 5

[3] The precipitate is colored a deep blue-black when excess titanous reagent is present. With high concentrations of nitro compounds, the titanous reagent is oxidized to the titanic form, and a white precipitate appears on the addition of alkali.

[4] Na_2CO_3 can be used in place of NaOH for precipitation of the titanium reagent, but foaming occurs after reacidification due to production of CO_2.

[5] This ratio of solvent to aqueous phase was found to give minimum volume change due to the mutual solubility of the two phases. We have also used isopropyl acetate, first suggested to us by Dr. E. K. Marshall, Jr., with equally good results.

ml. of the ethyl acetate layer was transferred by pipette to a 20 ml. beaker and evaporated to dryness on a steam bath. The residue was dissolved in 3 ml. of water[6] and analyzed by the titanium reduction procedure. In addition, microbiological assays (7) were run on the original urine samples,[7] and colorimetric analyses were also made for aryl amines and total nitro compounds. The results presented in Table II show fair agreement between the microbiological and chemical extraction procedures. Data on the distribution of the purified metabolic products of chloramphenicol between ethyl acetate and aqueous buffers at various pH levels will be presented elsewhere.

TABLE II

Analysis of Dog Urine for Chloramphenicol

Urine samples collected from dogs which had been given chloramphenicol by mouth were analyzed for aryl amines and total aromatic nitro compounds by colorimetric methods, and for unchanged chloramphenicol by the microbiological and solvent extraction procedures. All results are expressed as γ of chloramphenicol-equivalents/ml. of urine.

Sample no.	Aryl amines	Total nitro compounds	Chloramphenicol	
			Extraction	Microbiological
46	600	190	5	4
33	340	420	7	5
25	440	860	37	28
19	670	2030	130	120
40	720	3700	130	120
10	570	3300	220	220
17	460	5220	240	230
21	270	1820	240	230
23	280	5980	440	380
22	280	6350	600	610
43	420	3760	560	510

Interfering Substances

A survey of normal tissue constituents showed that adenine, adenosine triphosphate and nucleic acids, after heating with zinc in acid solution, formed derivatives which would diazotize and couple with the Bratton-Marshall reagent, but these compounds did not react in

[6] With blood and tissue specimens the solution may be turbid at this point due to the presence of lipide substances, which can be eliminated by extraction with an equal volume of petroleum ether.

[7] The microbiological assays were conducted by Dr. J. L. Schwab and Mrs. Margaret Galbraith, to whom we express our appreciation.

this manner after treatment with titanous chloride. Both the zinc and titanium procedures were found to yield colored derivatives with folic acid by reduction of the —NH—CH_2— linkage to form a primary amine. The zinc method described in this paper has been applied to the determination of adenine, and the titanium method has been found suitable for the determination of folic acid (12).

Acknowledgments

We are especially indebted to Dr. A. C. Bratton, Jr., for his constant help and advice during the course of this work; to Dr. Quentin R. Bartz and Dr. John Ehrlich for a sample of crystalline chloramphenicol; and to Dr. Harry Crooks for preliminary data on the structure of chloramphenicol during the early stages of the work.

Summary

A colorimetric procedure is described for the determination of chloramphenicol in biological materials, based on the reduction of the aromatic nitro group to a primary amine with titanous chloride or metallic zinc, followed by diazotization and coupling with the Bratton-Marshall reagent. This determination includes certain inactive metabolic derivatives of chloramphenicol which are shown to be present in high concentration in urine, as well as active chloramphenicol. The method can be made specific for chloramphenicol by preliminary extraction with organic solvents.

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Factors Influencing Fat Synthesis by *Rhodotorula gracilis*

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Introduction

The production of fat by microorganisms has long been a subject of interest to research and to industry. Attempts to produce fat on a commercial scale by the growth of microorganisms were made in Germany during both World Wars. *Endomyces vernalis* was employed during World War I and *Oospora lactis* during World War II (1,6). It is doubtful whether they were successful either time because the enormous surface areas required by both of these organisms undoubtedly presented overwhelming technical difficulties when commercial scale cultivation was attempted.

However, recent investigations have shown that there are some yeasts and molds capable of synthesizing significant amounts of fat when grown in submerged cultures; Fusaria (3,10,21), Mucor and molds related to it (4), were among the first to be noted. Certain Torula yeasts, *e.g.*, *Torulopsis lipofera* (9) and *Rhodotorula gracilis* (5), and the yeast *Nectaromyces reukaufii* (13) have also been found capable of synthesizing significant amounts of fat under conditions of submerged culture. The soil yeast isolated by Starkey (19) is unique in that it can grow and synthesize fat in media almost devoid of nitrogen. Since submerged culture techniques are more applicable to industrial operations it was deemed advisable to investigate this method of producing fat in some detail in order to evaluate its economic potentialities.

This paper deals mainly with the synthesis of fat by *Rhodotorula gracilis* grown in submerged cultures on a molasses medium; however, some experiments were made with glucose for purposes of comparison. *Rh. gracilis* has been reported to be one of the most efficient of the fat synthesizing organisms which can be grown in submerged cultures. By theoretical reasoning, Rippel (14) concluded that the fat coefficient—or grams of fat synthesized from 100 grams of carbohydrate utilized—could hardly exceed 15. However, Enebo, Anderson and Lundin (5) have shown that *Rh. gracilis* can attain a fat coefficient value as high as

16–18 when inverted commercial sucrose is used as a source of carbohydrate.

Efficient aeration and proper nitrogen supply are known to be salient factors in microbial fat synthesis. Smedley-MacLean *et al.* (16,17) have made thorough studies of these factors with brewers' yeast, and Heide (8) and Raaf (12) have reported similar studies with *Endomyces vernalis* The reviews by Smedley-MacLean (18), Bernhauer (2) and Kleinzeller (9a) give detailed discussions of the factors involved in fat synthesis. However, data for the submerged cultivation of fat producing organisms on a molasses medium were lacking. Since molasses offers one of the most economical growth media, these variables were considered worthy of investigation.

Experimental Procedure

Organisms

The culture of *Rh. gracilis* used in these experiments was obtained from Dr. H. Lundin of the Royal Technical University, Stockholm, Sweden. Other fat synthesizing organisms were also evaluated, including *Endomyces vernalis*, *Torulopsis pulcherrima*, *Oospora lactis*, *Torulopsis lipofera*, *Candida reukaufii*, and Starkey's soil yeast[1] (19). However, *Rhodotorula gracilis* was found to be far superior to any of these organisms, both in fat content and fat coefficient. The fat content of Starkey's soil yeast was found to be comparable to *Rh. gracilis* but the fat coefficient and rate of sugar utilization were much lower. Therefore, *Rh. gracilis* was employed in all the experiments reported in this paper.

Culture Methods

Formulas for the 3 media that were used in these studies are given in Table I. Yeast cultures were transferred once a week on agar slants containing glucose (Medium II) and molasses (Medium III). After 48–72 hr. incubation at 28°C., these slants were used as inocula for the fat synthesis experiments. The surface growth from an agar slant was suspended in sterile water immediately before inoculation. The inoculated medium contained approximately 5 million cells/ml. This type of inoculum was found to be just as satisfactory as that obtained from aerated or shaken cultures.

Cultivation Procedure

In cultivation utilizing glucose as the sugar source, the glucose was sterilized separately and added to the clarified, sterilized basal medium. All glucose media were

[1] These cultures were obtained from Professor E. McCoy of the University of Wisconsin, Madison, Wisconsin, Dr. L. J. Wickerham of Northern Regional Research Laboratory, Peoria, Illinois, and Dr. R. L. Starkey of Rutgers University, New Brunswick, New Jersey.

TABLE I

Composition of Media[a]

Medium[b] I		Medium II		Medium III	
	g./l.		*g./l.*		*g./l.*
$(NH_4)_2SO_4$	1.0	KH_2PO_4	1.0	$(NH_4)_2SO_4$	1.0
KH_2PO_4	1.0	Yeast extract (Difco)	5.0	KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	1.0			Carbohydrate[d]	20.0
NaCl	0.5	Carbohydrate[d]	20.0	pH	4.6–4.8
$CaCl_2 \cdot 6H_2O$	0.5	pH	6.0		
$FeCl_3 \cdot 6H_2O$	0.005				
Yeast extract (Difco) or malt extract (Difco)[c]	1.5				
Carbohydrate[d]	Varying				
pH (with H_2SO_4)	4.6–4.8				

[a] All salts and glucose were of C. P. grade. All media were sterilized at 15 p.s.i. for 30 min.

[b] This medium was essentially the same as that employed by Enebo *et al.* (5).

[c] Yeast extract and malt extract were found interchangeable. The yeast extract contained 8.8% nitrogen.

[d] Carbohydrate refers to either glucose or sugars contained in molasses. Blackstrap molasses obtained from U. S. Sugar Corp., Clewiston, Fla., was used throughout the present experimentation. This molasses had a sp. gr. of 1.44 (25°C.), a total sugar content of 46.5%, and a nitrogen content of 1.0%.

buffered with sterile $CaCO_3$ (0.5 g./l.) to check the drop in pH caused by the formation of H_2SO_4 from $(NH_4)_2SO_4$. In cultivation utilizing molasses, the medium was prepared as follows: sufficient water was added to 200 g. of molasses to make a total volume of 500 ml. The diluted molasses was centrifuged at 2500 r.p.m. for 15 min. to remove the suspended insoluble matter. Aliquots of the centrifugate[2] were added to solutions of the nutrient salts and the resulting medium was sterilized at 15 p.s.i. for 30 min. Separate sterilization of the dilute molasses and nutrients gave the same results as when all the ingredients were sterilized together. Unless stated otherwise, the media contained 4% sugar (either glucose or total sugar as glucose after hydrolysis). After inoculation, one drop of Foamicide A[3]/100 ml. of medium was added to suppress foaming.

Aeration experiments were made in 2 l., cone-shaped flasks, each containing 300 ml. of medium; shake cultures were grown in 500 ml. Erlenmeyer flasks, each containing

[2] The centrifugate still contained traces of insoluble solids. They were determined in uninoculated blank tests. Corrections were then applied to the dry weight of yeast, usually less than 2%.

[3] Wyandotte Chemicals Corp.

80 ml. of medium. Humidified sterile air was passed into the media through Aloxite gas diffusers. The rate of aeration was measured by a wet-test gas meter. Unless stated otherwise, 0.5–0.8 volume of air/volume of medium/min. was used. In shaking experiments, a shaker with a stroke length of 4 cm. was operated at 100 cycles per min. All flasks were incubated at the optimum temperature of the yeast, 27–29°C.

Samples were taken periodically during cultivation and analyzed for sugar. When all available sugar was utilized, the resulting medium was analyzed for dry weight of yeast and for total fat content. The yeast population was determined by means of a Neubauer counting chamber; the culture was checked for contamination at the same time.

Analytical Procedures

Dry Yeast

A 15 ml. sample was diluted to 40 ml. and centrifuged at 3500 r.p.m. in a weighed 40 ml. Pyrex centrifuge tube. The cells were washed once with $M/15$ KH_2PO_4 (7). After drying for 18–24 hr. at 60°C. under a vacuum of 27–28 in. Hg, the tubes were weighed again. Drying to constant weight at 110°C. resulted in less than 2% additional loss in weight.

Reducing Sugar

Sugars were determined by the micro method of Shaffer and Somogyi (15). The molasses medium was analyzed for total sugar as glucose after hydrolyzing in 0.35 N HCl for 5 min. in a boiling water bath.

Fat

A method for fat determination suggested by Gray (7) and based on one by Smedley-MacLean (16) was followed. The dried yeast cells were hydrolyzed in 1 N HCl for 2 hr. in a boiling water bath, filtered, dried and extracted for 8 hr. with anhydrous ether in a Goldfisch extractor. It was later found that fat analyses could be obtained satisfactorily by hydrolyzing the yeast cells directly in the medium, which had been acidified to 1 N with HCl.

Experimental Results

Preliminary Experiments in Glucose Media

Fat synthesis was first studied using a glucose medium (Medium I) in order to have some means of evaluating the results obtained with molasses media. The results agreed in general with those reported by Enebo *et al.* (5). *Rh. gracilis* was found capable of completely utilizing 4 g. of glucose/100 ml. of medium in 4 days, producing over 30% of dry yeast (based on glucose utilized) with a fat content of 60% (based on dry yeast). The unusually high fat coefficient of 16 to 18 was also con-

firmed. Data from a typical experiment in a glucose medium is presented in Expt. 1, Table II.

Analysis of fat content and estimations of cell population during cultivation confirmed the earlier view that the process can be divided into two phases, namely, a phase of protein formation, *i.e.*, cell multiplication, followed by a phase of fat formation (6,11). During the first 24 hr. there was a rapid increase in cell population (from 5 millions/ml. to 600 millions/ml.) when practically no fat was synthesized. In the following 3 days the fat accumulated steadily while the cell population remained practically constant.

TABLE II

Fat Synthesis in Molasses vs. Glucose Media[a]

Expt. no.	Sugar substrate	Medium	Sugar utilization (based on initial sugar)	Yield of dry yeast (based on sugar utilized) (*A*)	Fat content of dry yeast (*B*)	Fat coefficient $(A) \times (B)/100$
			per cent	*per cent*	*per cent*	
1	Glucose	Medium I[b]	97.7	31.2	59.0	18.4
2	Glucose	Medium I[b] with Y.E.[c] left out	61.5	23.1	56.7	13.1
3	Molasses	Medium I[b]	87.0[d]	40.6	28.3	11.5
4	Molasses	Medium I[b] with Y.E.[c] left out	86.8[d]	39.2	31.3	12.3
5	Molasses	$(NH_4)_2SO_4$ 1 g./l. KH_2PO_4 1 g./l.	88.2[d]	37.8	35.9	13.6
6	Molasses	No nutrient added	56.0	37.4	47.6	17.7

[a] Cultivation was carried out with aeration by sparging for 4–6 days. Initial sugar concentration was 4% in all cases.

[b] Medium I contained yeast extract, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, NaCl, $CaCl_2 \cdot 6H_2O$, and $FeCl_3 \cdot 6H_2O$.

[c] Y.E. denotes yeast extract.

[d] Maximum sugar utilization was 90% of initial sugar. These figures represent complete utilization.

The effect of the concentrations of nitrogen nutrients, such as $(NH_4)_2SO_4$ and yeast or malt extract, upon fat synthesis also agreed with many previous reports (5,8,12,19). Media rich in nitrogen support an abundant growth of yeast but the fat content is always low. However, as pointed out by Enebo *et al.* (5), there is an optimum concentration of nitrogen which must be furnished if the organisms are to synthesize the maximum amount of fat. Experiments with glucose showed that the optimum concentration of nitrogen can be furnished by 1 g. of $(NH_4)_2SO_4$ plus 1.5 g. of yeast extract/l. of medium.

The report by Enebo *et al.* also emphasized the importance of neutralization during cultivation. The H_2SO_4 liberated from $(NH_4)_2SO_4$ lowers the pH of the medium markedly, resulting in an inactivation of the yeast. In the present investigation, the addition of a small amount of $CaCO_3$ was found convenient for neutralizing the acid formed.

Comparison of Fat Formation in Molasses and Glucose Media

Experiments with molasses media show that molasses apparently contains appreciable amounts of the nutrients that are required by the yeast. Total cell population and yield of dry yeast were higher in molasses media than in glucose. A population of over one billion cells/ml. was attained in molasses media as against 500–600 millions/ml. in glucose. Table II presents a comparison of growth and fat synthesis in these two kinds of media. This table also shows that omission of yeast extract from molasses media had no effect on growth and fat synthesis but there was a marked reduction in the yields of yeast and fat when it was omitted from glucose medium. With $(NH_4)_2SO_4$ and KH_2PO_4 as the only nutrients added to a molasses medium (Expt. 5) the yeast grew as well and synthesized fat even better than when grown in a molasses medium containing all the added nutrients of Medium I (Expt. 3). Molasses, without any added nutrients, supported fairly good growth although the sugar utilization was incomplete (Expt. 6). With the exception of the medium containing only molasses (Expt. 6), the nutrients present in the molasses media produced greater yields of yeast but the fat content was considerably less than from glucose media. From these results it may be concluded that it is possible to obtain complete sugar utilization from molasses media without reducing the yield of fat if the proper concentrations of $(NH_4)_2SO_4$ and KH_2PO_4 are present.

The pH of a molasses-water mixture containing 4% sugar was 4.6–4.7; therefore, no initial pH adjustment was necessary. During cultivation, the pH of the medium gradually rose to 5.6–6.2 and consequently no neutralization was required. This rise in pH was probably due to the utilization by the yeast of organic acid salts present in the molasses media.

Unlike glucose, utilization of sugar in a molasses medium always stopped when 88–90% of the initial sugar was consumed. Apparently the residual 10–12% consisted of non-fermentable sugar and other reducing substances which analyzed as sugars.

Effect of KH_2PO_4 and $(NH_4)_2SO_4$ upon Fat Synthesis from Molasses

To find the optimum concentrations of $(NH_4)_2SO_4$ and KH_2PO_4 for fat synthesis in molasses media, experiments were performed with varying concentrations of these two compounds, added separately and in combination. Cultures supplemented in this manner were tested, using both aerated and shake flask cultures.

As shown in Fig. 1, KH_2PO_4 alone improved the sugar utilization to a limited extent (from 50.2% to 62.0%) but had no effect on the fat coefficient. Fig. 2 shows that $(NH_4)_2SO_4$ was very effective in improving sugar utilization; the yield of yeast increased slightly at higher $(NH_4)_2SO_4$ concentrations and no appreciable reduction was observed in the yield of fat with a concentration of $(NH_4)_2SO_4$ as high as 2 g./l. of medium. However, 3 g./l. caused a marked decline in both fat content and fat coefficient, and sugar utilization was incomplete. Presumably, the well nourished yeast lost some of its fat-synthesizing power and growth stopped before the sugar and $(NH_4)_2SO_4$ were completely utilized because other nutrients or growth factors had been exhausted.

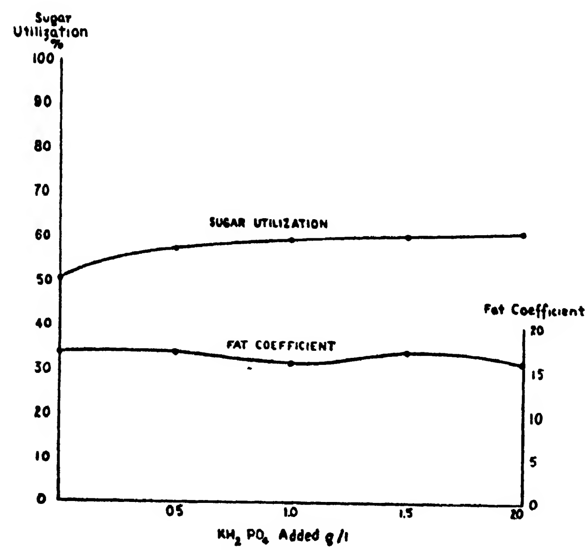

FIG. 1. Effect of KH_2PO_4 on fat synthesis in molasses medium. Cultivations were made in flasks with sparged air and in shake flasks, with KH_2PO_4 as the only nutrient added to the medium. Initial sugar concentration, 4 g./100 ml. of medium. Cultivation was stopped after 48–60 hr., since longer periods of time did not increase the amount of sugar utilized.

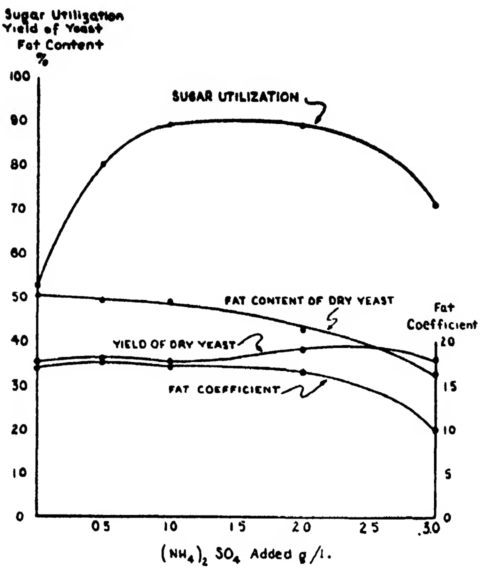

FIG. 2. Effect of $(NH_4)_2SO_4$ on fat synthesis in molasses medium. Cultivations were made in flasks sparged with air and in shake flasks, with $(NH_4)_2SO_4$ as the only nutrient added to the medium. Cultivation was stopped after 48–60 hr. Initial sugar concentration, 4 g./100 ml. of medium.

Table III shows the effect of $(NH_4)_2SO_4$ and KH_2PO_4 added in combination to the medium. It was found that 0.5 g. of $(NH_4)_2SO_4$/l. was adequate to effect complete sugar utilization when 0.5 g. of KH_2PO_4 was also added (Expt. 4). As little as 0.25 g. of $(NH_4)_2SO_4$/l. would bring about complete sugar utilization in the presence of 1 g. of KH_2PO_4/l. (Expt. 8). Apparently, the presence of KH_2PO_4 enhanced the effect of $(NH_4)_2SO_4$. On the other hand, 1.5 g. of $(NH_4)_2SO_4$/l. was high enough to cause a decline in fat yield if an equal amount of KH_2PO_4 was added (Expt. 13). It was found that when higher concentrations of these two compounds were used, the yield of fat was further reduced (Expts. 6 and 12). It is interesting to note that with 1.0 g. each of $(NH_4)_2SO_4$ and KH_2PO_4/l., the fat coefficient showed remarkably wide variations. Results of two extreme cases are shown in Expts. 10 and 11. Such variation is to be expected, since this concentration of the two salts approaches the upper limit for efficient fat synthesis. It is possible that phosphate enables the yeast to utilize more of the nitrogen contained in molasses since the addition of phosphate markedly enhanced the effect of $(NH_4)_2SO_4$.

Effect of Aeration upon Fat Synthesis

Varying the rate of aeration from 0.1 to 1 volume of air/min./volume of medium had no effect on the yields of yeast or fat. Aeration by shaking proved to be just as effective as by sparging, but stationary cultures produced very inferior results (see Table IV). The effect of aeration on the rate of sugar utilization will be discussed later.

TABLE III

Effect of $(NH_4)_2SO_4$ and KH_2PO_4 on Fat Synthesis in Molasses Media[a]

Expt. No.	KH_2PO_4 added	$(NH_4)_2SO_4$ added	Sugar utilization (based on initial sugar)	Yield of dry yeast (based on sugar utilized) (*A*)	Fat content of dry yeast (*B*)	Fat coefficient $(A)\times(B)/100$
	g./l.	g./l.	per cent	per cent	per cent	
1	0.0	0.0	60.2[b]	37.8	46.9	17.8
2	0.5	0.0	68.0[b]	38.2	47.1	18.0
3	0.5	0.25	75.0[b]	39.0	46.3	18.0
4	0.5	0.5	88.5	37.6	48.3	18.3
5	0.5	1.0	88.5	36.8	46.7	17.3
6	0.5	2.5	89.0	41.0	20.9	8.6
7	1.0	0.0	72.0[b]	36.5	49.7	18.2
8	1.0	0.25	88.0	37.6	49.2	18.5
9	1.0	0.5	88.4	37.0	48.7	18.0
10[c]	1.0	1.0	88.6	36.2	46.8	17.0
11[c]	1.0	1.0	88.9	37.4	32.6	12.2
12	1.0	2.0	89.0	40.5	24.2	9.8
13	1.5	1.5	88.9	38.8	28.3	11.0

[a] Cultivation was carried out in both flasks with sparged air and shake flasks with $(NH_4)_2SO_4$ and KH_2PO_4 as the only added nutrients. Initial sugar concentration was 4%. Cultivation was stopped at the end of 48–60 hr., beyond which practically no more sugar was consumed. Results are averages of at least two experiments.

[b] These figures indicate incomplete sugar utilization.

[c] Data of two extreme cases are listed here to show the wide variation in results.

Fat Synthesis at Higher Sugar Concentrations

All of the above experiments were made with media containing 4% sugar. However, it was considered advisable to determine whether the same high fat yields could be maintained using higher levels of sugar. It was found that *Rh. gracilis* could completely utilize 6 or 8 g. of sugar/100 ml., in either molasses or glucose media, and yields of yeast and fat, based on sugar utilized, were just as high as with lower concentrations (Table V).

TABLE IV

Effect of Aeration upon Fat Synthesis[a]

Aeration[b] ratio	Cultivation period	Sugar utilization (based on initial sugar)	Yield of dry yeast (based on sugar utilized) (*A*)	Fat content of dry yeast (*B*)	Fat coefficient (*A*)×(*B*)/100
	hr.	*per cent*	*per cent*	*per cent*	
1:1	40	89.7	36.5	49.6	18.1
1/3:1	60	88.5	36.6	48.8	17.9
1/8:1	60	88.8	37.2	48.2	17.8
0.1:1	72	88.7	36.2	48.6	17.6
Stationary culture[c]	12 days	22.0	40.0[d]	30.2	12.2[d]
Shaken culture	60	88.7	37.4	48.8	18.3

[a] Cultivation was carried out in molasses medium with 1 g. $(NH_4)_2SO_4$/l. as added nutrient. Initial sugar concentration was 4% in each case.

[b] Aeration ratio denotes the ratio, volume of air per min.: volume of medium.

[c] 100 ml. of the medium were used in a 500 ml. Erlenmeyer flask.

[d] Since the sugar utilization was very incomplete, experimental error in the yields must be high.

TABLE V

Fat Synthesis at Higher Sugar Concentrations in Molasses Media[a]

Initial sugar	$(NH_4)_2SO_4$ added	Cultivation period	Sugar utilization (based on initial sugar)	Yield of dry yeast (based on sugar utilized) (*A*)	Fat content of dry yeast (*B*)	Fat coefficient (*A*)×(*B*)/100
g./100 ml.	*g./l.*	*hr.*	*per cent*	*per cent*	*per cent*	
4.40	1	60	89.0	34.3	52.5	18.0
4.40	2	60	89.0	38.6	43.8	16.9
6.65	1	85[b]	60.3[d]	—	—	—
6.65	2	85[b]	89.2	34.8	46.1	16.0
8.80	1	108[c]	88.8	35.9	50.4	18.2
8.80	4	108[c]	89.3	35.9	41.6	14.8[e]

[a] Cultivation was carried out in shake flasks.

[b] There was an initial lag period of 24 hr. (see Fig. 5).

[c] There was an initial lag period of 48 hr. (see Fig. 5).

[d] Sugar utilization stopped before completion, probably due to contamination.

[e] Low fat coefficient was obviously due to high nitrogen content.

Factors Influencing the Rate of Sugar Utilization

In nearly all experiments, the diminution in sugar content of the medium was determined by periodic analyses. Some of the results are shown in Figs. 3, 4, and 5, where sugar concentrations during cultivation are plotted against time.

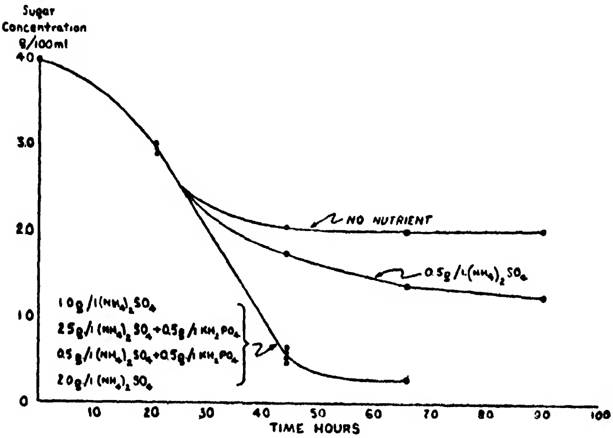

FIG. 3. Effect of nutrients on rate of sugar utilization in molasses medium. Cultivations were made in shake flasks.

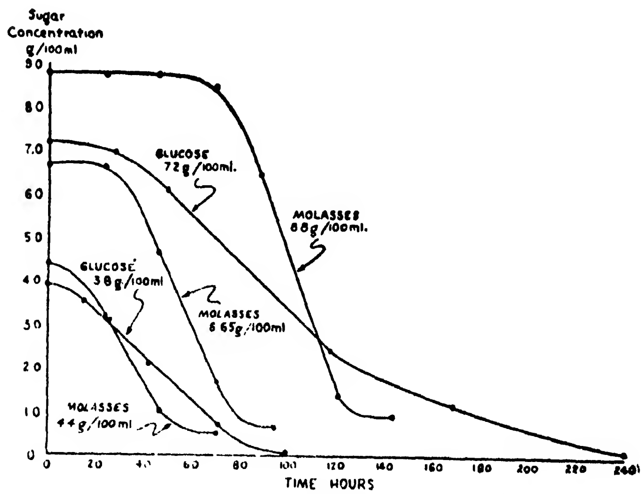

FIG. 4. Rate of sugar utilization at different initial sugar concentrations. Cultivations in glucose media were made in flasks sparged with air; shake flasks were used for cultivations made in molasses media.

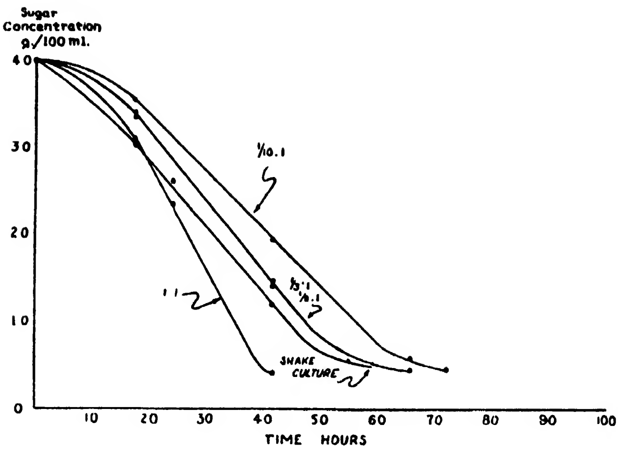

FIG. 5. Effect of aeration on rate of sugar utilization in molasses medium. Cultivations were made in molasses media containing 1 g./l. of $(NH_4)_2SO_4$ as added nutrient. Aeration is expressed as volume of air/volume of medium/min.

Fig. 3 shows that the rates of sugar utilization during the first 24–30 hr., in media containing 4% sugar, are the same in molasses media with or without the added nutrients—$(NH_4)_2SO_4$ and KH_2PO_4. Although sugar utilization in molasses media with insufficient nitrogen (0.5 g. of $(NH_4)_2SO_4$/l. or lower) failed to proceed to completion, the rates in all media which allowed complete sugar utilization were identical—independent of the amount of $(NH_4)_2SO_4$ and KH_2PO_4 added. The substances contained in molasses, rather than the added nutrients, evidently are the factors which govern the rate of sugar utilization.

At sugar concentrations higher than 4%, growth was not initiated until after a long lag phase, the length of which depended upon the initial sugar concentration (Fig. 4). No such phenomenon was observed in glucose media (Fig. 4). However, as soon as growth was initiated, the sugar was completely utilized within 48–60 hr. regardless of the initial sugar concentration. That is, the higher the concentration of molasses in the medium, the greater the rate of sugar utilization. Fig. 4 also shows that the rates of sugar utilization in molasses media were considerably higher than in glucose media at corresponding initial sugar concentrations. This acceleration of sugar utilization seems to conform with the observation that molasses media produced a greater number of cells and a higher dry weight of yeast than did glucose.

Aeration had a limited effect on the rate of sugar utilization. Curves

in Fig. 5 show that aeration ratios (volume of air/volume of medium/min.) of 1/3:1 and 1/8:1 produced identical curves for sugar utilization. The rate of sugar utilization was higher at an aeration ratio of 1:1, and lower at 0.1:1. Shake flask aeration was apparently equivalent to an air-sparging ratio of 1/3:1. Sugar utilization was extremely slow and incomplete in stationary cultures (Table IV). From a practical point of view, aeration ratios higher than 1/3:1 have no particular advantage.

Yeast Consumption of Fat Formed

It has been pointed out by many investigators that some microorganisms are capable of utilizing the fat stored in their cells as a source of energy (12,20); *Rh. gracilis* was found to be one of these. Table VI shows the diminution of fat which resulted from prolonged cultivation after all available sugar was exhausted. In both glucose and molasses media, marked reduction in the fat content occurred under such a treatment, amounting in 14 days, to 22.2% and 40%, respectively. A comparison of the loss in dry weight of yeast with that of fat indicates that fat is consumed in preference to other cell constituents. Therefore, to obtain the maximum recovery of fat, the yeast must be harvested as soon as sugar is completely utilized.

TABLE VI

Consumption of Fat on Prolonged Incubation[a]

Carbohydrate substrate	Incubation period	Dry weight of yeast		Fat		
		Amount found	Loss	Amount found	As content of dry yeast	Loss
	days	*g./100 ml.*	*per cent*	*g./100 ml.*	*per cent*	*per cent*
Glucose[b]	4[c]	1.23		0.810[c]	65.0	
	7	1.18	4.1	0.763	64.5	5.8
	14	1.04	15.4	0.630	60.6	22.2
Molasses[b]	3[c]	1.32[c]		0.625[c]	47.8	
	7	1.25	5.3	0.559	44.8	10.3
	14	1.06	19.2	0.375	35.4	40.0

[a] Cultivation was carried out with aeration by sparging, initial sugar was 4%.

[b] Medium I was used in experiments with glucose. 1.0 g. of $(NH_4)_2SO_4$/l. was used as added nutrient in the molasses medium.

[c] These values represent the results immediately after sugar was all consumed.

Discussion and Conclusion

Experimental data on growth and fat synthesis by *Rh. gracilis* in glucose media agree in general with many earlier reports on different fat-producing microorganisms. The importance of an optimum nitrogen concentration of the medium for maximum fat synthesis, emphasized by Enebo, Anderson and Lundin, has also been demonstrated in the present investigation. Under optimum conditions, the unusually high fat coefficient, 16–18, which was first reported by Enebo *et al.*, was confirmed.

Growth of *Rh. gracilis* and its fat synthesis in molasses media present many interesting features. The higher values for cell population, yield of dry yeast, and rate of sugar utilization for fat synthesis which were obtained in a molasses medium suggest that molasses contains substances which are nutritive or stimulatory to growth and increase the rate of fat synthesis of the yeast. The fact that the rate of sugar utilization is dependent upon the molasses concentration, rather than on added nutrients, *e.g.*, $(NH_4)_2SO_4$ and KH_2PO_4, further supports this assumption. The question remains, however, whether the higher rate of sugar utilization is a result of higher cell population, a stimulatory effect, or both, since no attempt was made to correlate these factors.

The presence of a lag phase in a molasses medium containing more than 4% sugar indicates the presence of substances which retard the initiation of growth. This retarding effect becomes more marked as the sugar concentration is increased. However, as soon as growth starts, this effect disappears and the stimulatory substances become functional.

Due to the presence of these nutritive substances in molasses, the influence of adding $(NH_4)_2SO_4$ and KH_2PO_4 becomes more critical than in a glucose medium. An insufficient supply of these two compounds in a molasses medium results in incomplete sugar utilization, while an excess causes a marked decline in fat yield. It appears that $(NH_4)_2SO_4$ has a direct effect, while KH_2PO_4 enhances the action of the former. A concentration of 1.0 g. of $(NH_4)_2SO_4$/l. alone, or 0.5 g. each of $(NH_4)_2SO_4$ and KH_2PO_4/l. proved to be satisfactory for complete sugar utilization and resulted in a fat coefficient which was as high as that obtained with a glucose medium, 16–18.

The increase of pH to 6 during growth in molasses media is of practical advantage since it simplifies the process by eliminating pH adjustment during cultivation.

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Summary

The unusually high fat coefficient of 16–18, and the importance of an optimum nitrogen concentration, which have been reported previously for *Rhodotorula gracilis* in a glucose medium, were confirmed. It was also shown that *Rh. gracilis* could be grown successfully in a molasses medium to produce fat with the same efficiency as in a glucose medium, provided proper amounts of nutrients—$(NH_4)_2SO_4$ alone or with KH_2PO_4— are added to the medium to insure complete sugar utilization.

It was found that certain substances present in molasses are capable of accelerating the sugar utilization and, therefore, the rate of sugar utilization is a function of the molasses concentration. This effect may be due to the increase in cell population under these conditions.

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Studies on the Mechanism of Action of Ionizing Radiations. III.[1] The Plasma Protein of Dogs after X-Ray Irradiation. An Electrophoretic Study

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Introduction

There have been several conflicting reports of altered concentrations in serum proteins following the administration of X-rays. In a series of human patients, Herzfeld and Schinz (1) reported a diminution of total protein and a decreased A/G ratio following therapeutic irradiation with X-rays. Wichels and Behrens (2) found similar changes in certain cases only, while Breitlander and Lasch (3) could find no significant variation in the blood proteins following irradiation. In an experimental study on dogs, Davy (4) found changes in the blood proteins following the administration of 500 roentgen units (r). There was an immediate fall in albumin, which returned to normal after 48 hr. Variations in the blood globulins were not consistent.

Results obtained by the earlier workers are difficult to interpret because the X-ray dosage was not given. Then, too, these and later workers were handicapped by the use of the inadequate salt fractionation methods for the determination of the serum proteins. With the method of electrophoresis as developed by Tiselius (5), the analytical difficulties have been largely overcome. It has been demonstrated that sera from any given animal species have a characteristic pattern (6,7). The dog, in particular, has been extensively studied (8).

This paper reports the effect of X-rays on the plasma protein pattern of dogs. The experiments were performed during the years 1944–45.

[1] Papers I and II of these studies have appeared in the Journal of General Physiology.

EXPERIMENTAL

Mongrel dogs were employed that had been treated with *"Lederle" antidistemper vaccine.* They were fed a stock diet consisting of Friskies, supplemented with ground meat twice weekly. X-rays, filtered with 0.5 mm. Cu and 1 mm. Al were administered in one total body dose from a 200 kv peak machine.

Control blood samples were drawn before the X-ray treatment and periodically thereafter until the animal died. Blood was collected in oxalated tubes and, after centrifugation, the plasma was withdrawn for analysis.

Total protein determinations were performed by a micro Kjeldahl digestion followed by nesslerization or distillation and titration. Non-protein nitrogen was determined on a filtrate obtained by precipitating the protein with trichloroacetic acid.

Electrophoretic analyses of the sera were carried out by the moving boundary method of Tiselius (5) in an apparatus equipped with the Schlieren scanning device described by Longsworth (9). Three to four cc. of serum or plasma were dialyzed for 24 hr. against 2 liters of veronal buffer (0.075 M, pH = 8.6).

Electrophoresis was carried out for 2 hr. in a long (11 cm.) analytical cell at 1.2°C., using a current of 23 milliamps and an E.M.F. of 310 v.

In one experiment (dog 128), samples of the terminal plasma obtained shortly before death were extracted with lipide solvents. One 10 cc. plasma sample was extracted by the modified Blix procedure (10) described by Zeldis *et al.* (11). A considerable amount of the dry protein failed to dissolve in the normal saline solution and electrophoretic analysis of the soluble portion showed that the greatest reduction in relative concentration had occurred in the β_2 complex fibrinogen fraction of the original plasma.

Another 12 cc. sample of the same plasma was lyophilized prior to extraction with lipide solvents. The dry, fluffy powder was extracted with two 80 cc. portions of absolute ethyl alcohol at −70°C. for 20 min. each, followed by one portion of alcohol-ether (7:3), and finally with two 80 cc. portions of absolute ether, all at −70°C. for 20 min. After removing the last traces of ether with a vacuum pump at room temperature, the resulting dry powder was readily soluble in normal saline solution.

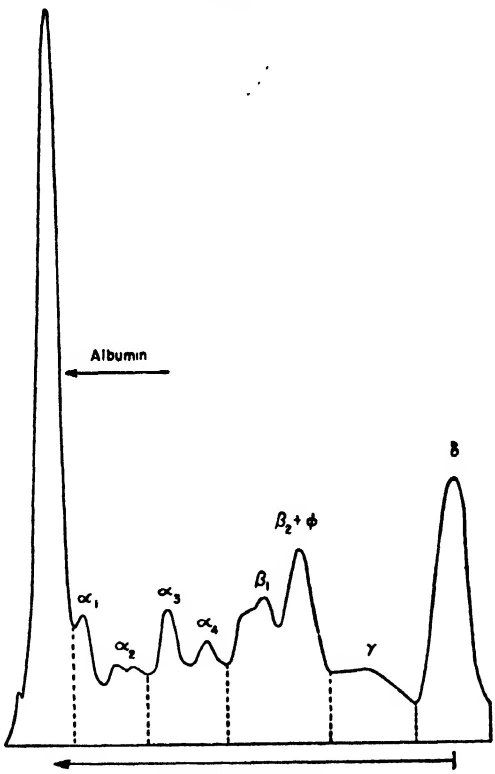
RESULTS

A typical normal pattern of the rising boundaries in dog plasma is shown in Fig. 1. In most dogs 8 distinct components may be distinguished. Following the nomenclature of Zeldis and Alling (8), they have been designated as α_1-, α_2-, α_3- and α_4-globulins; β_1-, β_2-globulins; fibrinogen (ϕ) and γ-globulin. The fibrinogen in dog plasma does not separate out as a distinct component; it migrates with the β_2-globulin and serves to increase this peak above that seen in serum.

Even these 8 components are not electrophoretically homogenous. As shown in Fig. 1. the α_2-globulin, as well as the β_1-globulin, has a split peak, indicative of inhomogeneity. In dogs dying of X-ray injury, the pattern becomes less complex, and many of the small peaks seen in the normal pattern appear to merge. Sometimes there are only 2 α-globulins

in the pathological sera. These seem to have the mobility of the α_2- and α_3-globulin of normal plasma. It will be shown that the concentration of α_3-globulin is significantly increased in injured dogs. The α-globulins have been divided into 2 fractions for purposes of calculation: α_1- and α_2-globulin are estimated together as well as α_3- and α_4-. In many cases β_1-globulin is poorly resolved and tends to merge with the β_2, ϕ component; consequently all 3 components were estimated together.

FIGURE I



Plasma Protein Changes Following a Single X-Ray Treatment

Protein changes in the sera of 5 X-ray treated dogs are shown in Table I. Dogs 44, 111 and 130 received a lethal dose of X-rays. They maintained their food intake for 7–8 days following X-ray treatment. Then they began to lose their appetite and stopped eating 3–4 days before death. Despite the maintained food intake, the plasma albumin

had already decreased 7 days after X-ray irradiation (dogs 111 and 130) At this time no significant changes were observed in any of the other components. Three to six days before death, these animals developed a fever which varied from 2 to 3°C. above normal. Coincident with this febrile condition there was a rise in the α_3-globulin component, which increased with each day that the dog survived. In general, there was also some increase in the β_2, ϕ-globulin fraction. The progressive changes in the plasma protein pattern are shown in Fig. 2. The α_3-globulin frequently exceeded the plasma albumin concentration (Fig. 3). In this terminal period, which occurs at 12–15 days after X-ray, the plasma albumin was decreased to less than half its normal value.

FIGURE 2
PLASMA PROTEIN PATTERNS ON DOG 128

FIGURE 2 (Part 2)

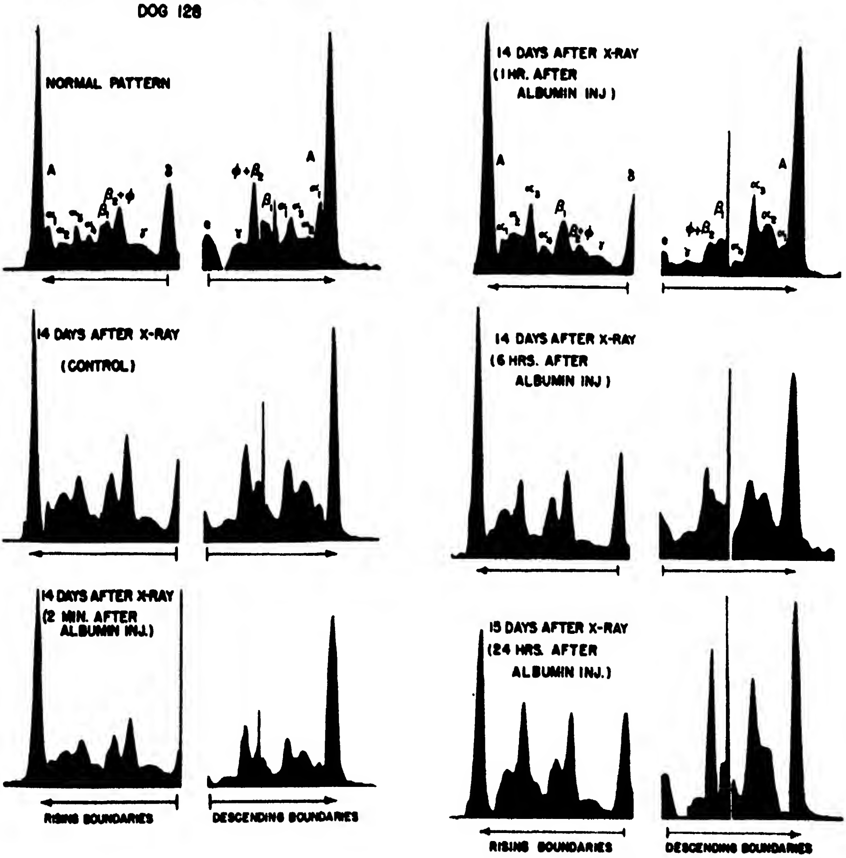
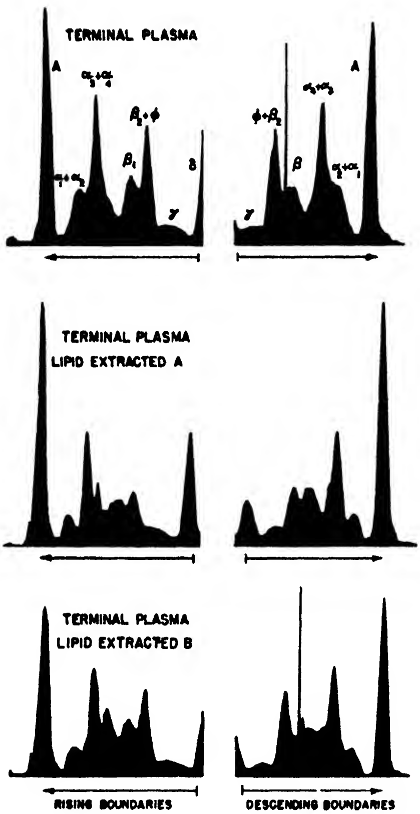


FIGURE 2 (Part 3)



Despite this albumin diminution, the total protein concentration was increased somewhat above normal. This must have been due entirely to the increased α_3- and β-globulins, since the plasma volume in this terminal period was also increased slightly above normal.

When normal dogs were starved for 3 or 4 days, no pronounced changes were observed in the plasma protein pattern. Hence, the alterations seen in X-rayed dogs are not due to the diminished food intake during the terminal period.

Dogs treated with a sublethal dose of X-rays did not show any marked changes in their plasma protein patterns (dog 36), or they had some changes 14 days after X-ray which returned to normal as recovery occurred (dog 24). On the other hand, a dog that died of distemper had,

TABLE I

Electrophoretic Analyses of Serum and Plasma Proteins on X-rayed Dogs

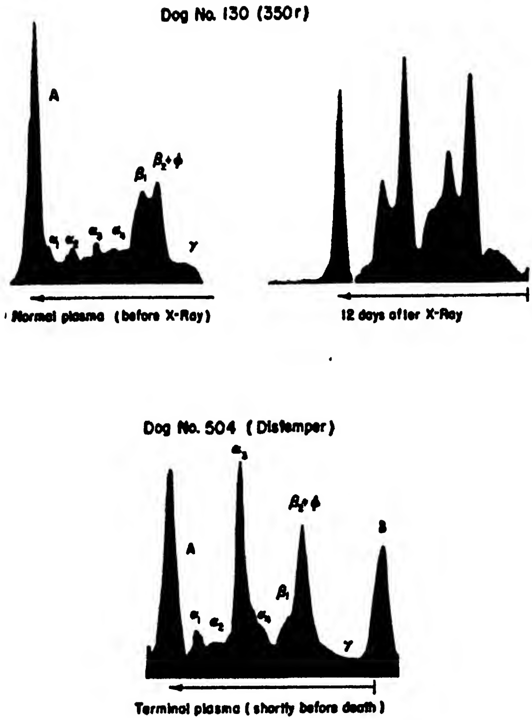
Dog no.[a] and X-ray dose	Serum or plasma	Days before or after X-ray	N.P.N. mg.-%	Total protein	Albumin	Globulins			
						$\alpha_1+\alpha_2$	$\alpha_3+\alpha_4$	$\beta_1+\beta_2+\phi$	γ
						Per cent of total protein			
44	Plasma	−10	36	6.08	44	11.7	14.3	18.9	11.1
300 r	Serum	−2	34	5.84	48	11.4	13.2	17	10.1
	Serum	+7	31	5.36	48.7	10.6	13.1	18.1	9.7
	Serum	+14	67	6.11	28	11.8	28.1	19.3	12.6
	Serum	+15	47	6.50	23.8	10.8	31.7	20.9	13.1
111	Plasma	−7	27	4.35	43.5	13.6	12.6	23.5	6.9
350 r	Plasma	−3	31	5.58	42	10.9	13.8	21.5	11.1
	Plasma	+7	39	5.13	38.6	16.6	11.7	22	11.5
	Plasma	+12	34	5.85	25.5	17.1	23.2	27.5	6.7
	Plasma	+13	97	6.43	16.3	11.2	22.6	44.7	5.3
130	Plasma	−7	28	5.64	40.2	8.2	10.8	35.8	5.1
350 r	Plasma	−3	32	6.20	41.5	5.0	8.9	37.6	6.8
	Plasma	+7	39	6.35	29.8	10.9	10.4	40.5	6.8
	Plasma	+12	50	6.78	19.2	13.9	23.4	35.5	8.1
	Plasma	+13	97	6.43	16.3	11.2	22.6	44.7	5.3
24	Serum	−8	—	6.13	42.4	16.3	6.5	32.6	3.3
250 r	Serum	−4	49	5.94	44.2	8.6	13.1	29.6	6.2
	Plasma	−4	49	6.38	44.2	8.3	9.7	29.6	6.3
	Plasma	+8	30	5.18	39.0	13.5	13.5	27.4	6.5
	Serum	+14	41	5.05	41.0	12.3	12.9	26.5	6.9
	Plasma	+14	42	5.37	34.8	13.4	13.4	29.8	8.4
	Plasma	+42	—	5.02	39.9	9.0	9.6	33.7	8.0
155	Plasma	0	27	5.86	45.1	14.0	14.0	17.6	9.0
400 r	Plasma	+6	25	5.62	37.2	13.5	16.0	24.9	8.3
	Plasma	+8	27	6.20	37.9	7.9	22.4	25.7	6.1
36	Serum	−8	24	5.82	51.8	13.2	11.2	16.8	7.0
200 r	Serum	−4	29	5.94	56.6	9.3	11.0	17.3	5.9
	Plasma	+14	—	5.22	47.0	12.3	13.0	28.0	8.2
504 (no X-ray)	Plasma	None	19	4.19	27.7	9.3	30.8	28.6	3.6

[a] Administered as a total body dose.

— Indicates number of days before X-ray.

+ Indicates number of days after X-ray.

FIGURE 3



in the terminal period, a plasma protein pattern that was very similar to that seen in dogs dying of X-ray injury (Fig. 3).

Effect of Injecting Bovine Albumin on the Abnormal Plasma Protein Pattern

Because of the reciprocal relationship between plasma albumin and α_3-globulin, it seemed desirable to see what effect replacement of the lost albumin would have on the plasma protein pattern. For these experiments crystallized bovine plasma albumin was used. Dogs were given a single lethal dose of X-rays (total body irradiation). When the animals had progressed to the terminal period, as evidenced by the onset of fever, the bovine albumin was injected. An amount of albumin equal to one-half of the total normal plasma albumin was dissolved in 25 cc. of 0.86% NaCl, and the entire solution was injected intravenously. Samples of blood were withdraxn 5 min., 1 hr., 6 hr., and 24 hr. after

TABLE II

Electrophoretic Analyses of Plasma Proteins on X-Rayed Dogs. Effect of Bovine Serum Albumin Injected in the Terminal Period

Dog no. and X-ray dose	Day and hour of sampling	N.P.N. mg.-%	Total protein	Albumin	Globulins			
					$\alpha_1+\alpha_2$	$\alpha_3+\alpha_4$	$\beta_1+\beta_2+\phi$	γ
					Per cent of total protein			
123	1-23-46	25.4	6.02	44.3	10.6	10.6	25.9	8.5
350 r	1-29-46	25.1	5.68	48.1	10.7	11.7	22.7	6.9
(X-rayed	2-6-46 (Control)	29.6	6.30	24.0	14.3	20.3	34.5	7.0
on	2-6-46 (1 hr.)	30.7	6.59	35.4	9.7	20.0	28.7	6.2
1-29-46)	2-6-46 (7 hr.)	26.8	5.86	33.3	9.4	20.6	30.0	6.7
	2-7-46	24.8	6.26	30.6	10.1	21.2	31.9	6.2
	2-8-46	32.0	5.88	24.3	10.0	23.1	36.4	6.1
	2-9-46	51.8	5.54	23.5	11.6	29.4	28.9	6.8
138	3-5-46	23.6	5.65	43.5	11.3	12.2	25.3	7.4
400 r	3-16-46 (Control)	29.8	5.77	20.6	11.6	26.7	35.9	5.2
(X-rayed	3-16-46(5 min.)	28.2	6.38	33.2	9.1	24.1	28.5	5.0
on	3-16-46 (1 hr.)	28.2	6.06	32.2	9.4	24.1	28.7	5.6
3-5-46)								
138	4-9-46	30.0	5.80	44.5	6.2	12.4	28.3	6.9
350 r	4-16-46	26.8	5.63	45.3	7.8	12.6	25.8	8.5
(X-rayed	4-23-46 (Control)	31.8	6.68	28.4	14.8	17.2	32.2	7.5
on	4-23-46 (2 min.)	32.6	7.23	37.1	11.9	16.3	27.0	7.7
4-9-46)	4-23-46[a] (1 hr.)	36.4	6.70	43.3	13.3	18.2	20.0	5.4
	4-23-46 (6 hr.)	35.2	6.53	36.1	13.2	17.9	27.9	4.7
	4-24-46	31.0	7.13	28.6	11.1	25.4	29.3	5.6
	4-24-46 (Terminal)	37.6	6.73	29.1	10.1	27.3	28.8	4.7
	(Terminal) Lipide extracted (a)	16.2	5.19	39.7	6.4	24.2	24.1	5.8
	(Terminal) Lipide extracted (b)	32.6	5.78	29.7	5.5	31.1	28.3	5.2
	5-13-46 (Control)	31	5.90	40.5	12	12.7	28.5	6.1
162	5 min.	29.8	6.20	53.1	10.3	10	21.3	5.3
(no	1 hr.	31.2	5.82	48.8	11	11.5	23.2	5.3
X-ray)	6 hr.	29.0	5.72	51.8	12.4	11.2	21.0	3.5

Dog 123—5.12 g. albumin in 25 cc. saline injected 2-6-46.
Dog 138—5.22 g. albumin in 25 cc. saline injected 3-16-46.
Dog 128—4.3 g. albumin in 25 cc. saline injected 4-23-46.
Dog 162—6.27 g. albumin in 25 cc. saline injected 5-13-46.

[a] Some clotting occurred in this sample, thereby lowering the fibrinogen fraction and raising the albunin out of proportion to the other components.

the injection and analyzed electrophoretically. A similar experiment was performed on a normal dog.

As shown in Table II, the immediate effect of the albumin injection was to elevate the dog's albumin fraction and to decrease the concentration of the β complex-fibrinogen fraction. The total amount of albumin injected was almost completely recoverable when analyses were made within 5 min. after injection. This was true of the normal dog, as well as of X-rayed animals. The per cent of injected albumin found at various time intervals after injection is given in Table III. Calculation of extra plasma albumin is based on the assumption that the volume of saline injected with the bovine albumin remained in the circulating blood following the injection.

TABLE III

Recovery of Injected Bovine Plasma Albumin at Various Times after Injection

Dog no. and treatment	Per cent of injected albumin found in the plasma[a]			
	5 min. after inj.	1 hr. after inj.	6 hr. after inj.	24 hr. after inj.
123 (350 r)		81	47	36
128 (350 r)	91		58	26
138 (400 r)	98	82		
162 (no X-ray)	94	53	64	

[a] *Calculation*

[Δ] Plasma volume (X-rayed dogs) = 6.2% of body weight.

Plasma volume (normal dog) = 5.7% of body weight

$$\text{Per cent of injected albumin} = \frac{AV_2 \text{ (after injection)} - AV_1 \text{ (before injection)}}{a} \times 100.$$

Where: A = Albumin concentration, g. per 100 cc., from electrophoretic pattern.

V_1 = Plasma volume, $\frac{\text{cc.}}{100}$.

V_2 = Plasma volume, $\frac{\text{cc.}+25}{100}$.

a = g. of bovine albumin injected.

[Δ] Determined on a series of 6 normal dogs and 6 X-rayed dogs with Evans' blue dye.

In two of the three X-rayed dogs, the β complex-fibrinogen component decreased after the albumin injection. This occurred also in the normal dog. On the other hand, the concentration of the elevated α_3-globulin was unaffected by the albumin injection. The normal dog, as well as the X-rayed dogs, lost approximately 50% of the injected albumin within 6 hr. This was not lost through the kidney. Dog No. 128 was catheterized during the 6 hr. period following the injection of albumin; the urine obtained in this period contained some protein, but a total of only 55 mg. was excreted.

Extraction of Lipides from the Terminal Plasma

Zeldis *et al.* (11) have reported that the elevation of total globulin observed in hypoproteinemic, hyperlipemic dogs is largely due to the lipemia these animals developed. However, their electrophoretic patterns show that the α_3-globulin is not reduced by lipide extraction. The marked reduction occurs in the α_1- + α_2-globulin.

It was of interest to see whether the greatly increased α_3- + α_4-globulin component in the terminal plasma of X-ray injured dogs could be reduced by lipide extraction. A 10 cc. sample of plasma from dog No. 128 was extracted, using the procedure described by Zeldis *et al.*, except that all the extractions were carried out at the temperature of an acetone-solid CO_2 mixture, approximately $-70°C$. The dry powder obtained by this procedure was not completely soluble in *N* NaCl. A considerable amount of a gelatinous precipitate remained insoluble. From the electrophoretic pattern, *Fig. 2*. terminal plasma (lipide-extracted a), it can be seen that the insoluble material was derived chiefly from the β complex-fibrinogen fraction. The α_3- + α_4-globulin fraction was hardly affected by this treatment.

As described in the experimental part of this paper, another 12 cc. sample of the same plasma was extracted, using another procedure. As shown in *Fig. 2*, terminal plasma (lipide-extracted b), there is no abnormal diminution of the β complex-fibrinogen fraction in this extracted plasma. Table II shows that the α_3- + α_4-globulin was not diminished by lipide extraction, but that α_1- + α_2-globulin was decreased to one-half its original concentration.

These results indicate that the elevated α_3- + α_4-globulin concentration in the terminal plasma of X-ray injured dogs is an increase in true protein and is not due to an elevation in the lipide content.

Discussion

There was no characteristic alteration in the electrophoretic pattern of a dog's plasma proteins immediately after treatment with a lethal total body dose of X-rays. After such a treatment dogs survived for 12–18 days. They maintained their food intake for about one week after X-ray and no consistently abnormal change in the plasma electrophoretic pattern was demonstrable other than a slight decrease in albumin. During the second week after X-ray, plasma albumin diminished markedly and, with the onset of fever, which occurred from 3 to 5 days before death, there was developed a striking change in the electrophoretic pattern. α_3-Globulin increased and this was usually accompanied by an increase in the β complex fraction. One to two days before death this α-globulin frequently exceeded the albumin. It is not known whether the diminution in albumin is due to impaired synthesis of this protein, or whether it is lost from the blood because of increased capillary permeability. It is not lost in the urine.

This α-globulin increase seems to differ from that described by Zeldis *et al.* (12) which occurs in chronically depleted, hypoproteinemic dogs. In those dogs all the α-globulins increased, particularly the α_1-globulin. Furthermore, these authors demonstrated that the increase in α-globulins, as determined electrophoretically, is due to an increase in plasma lipides, and very little if any increase in protein N occurs. Extraction of these lipides (10) reduced α_1- and α_2-globulin to the normal level. However, their electrophoretic patterns show that this procedure had little effect on the concentration of the other α-globulins.

In the dogs dying of X-ray injury, the greatly increased α_3- + α_4-globulin fraction is a true protein fraction. Its concentration, as determined by electrophoresis, is not altered by lipide extraction.

It is tempting to consider that the elevated globulin is brought about in order to maintain the colloid osmotic pressure in the blood which would otherwise be diminished due to the loss of albumin. If this were true, replacement of the albumin might tend to bring about a diminution of the elevated globulins. In the experiments described, this did not occur; however, a single injection of albumin could hardly be expected to accomplish this result. It would be interesting to observe the effect of frequent injections of homologous albumin.

The immediate cause for the death of X-ray injured dogs is not well

understood. In some respects the terminal stage resembles a shock syndrome, but it may be that the dogs die of an overwhelming infection which is permitted to run rampant because of the previous destruction of the lymphoid tissues and leucocytes. It has not been possible to keep the dogs alive for more than 5 days after the onset of fever. If some treatment could be found and death delayed for days or perhaps weeks, these X-rayed dogs would provide excellent material for a careful study of the α_3-globulin.

Summary

The administration of a lethal amount of X-rays administered as a total body dose to dogs produced no characteristic change in the plasma protein pattern for one week following the treatment. From the seventh day after X-ray until death of the animal, the albumin diminished steadily. Coincident with the onset of fever, there was a striking increase in α_3-globulin and some increase in the β-globulin, fibrinogen fraction. Despite an increased blood volume in the terminal period, the total protein N was somewhat increased, a reflection of the sharp rise which is observed in the globulins. The increase in plasma globulin by electrophoretic analysis was not due primarily to an increase in plasma lipides. The injection of bovine albumin had no effect other than to raise the plasma albumin temporarily.

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On the Mechanism of Enzyme Action. XXXIX. A Comparative Study of the Metabolism of Carbohydrates, in the Presence of Inorganic and Organic Phosphates, by *Merulius lacrymans* and *Marasmius chordalis*

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Introduction

In a previous communication from this laboratory (15), studies have been presented of the metabolism of the molds *Merulius lacrymans* (Melac) and *Marasmius chordalis* (Machor) on media containing glucose, as well as sorbitol, adonitol, isopropyl alcohol and ethyl alcohol.

Since the main function of both of these molds in nature is cellulose destruction, Melac causing a dry rot on domestic wood, and Machor as a humus former on ground litter, leaving lignin behind, it was suggested by Dr. Nord that a comparison should be made of the metabolism of these molds on cellulose, cellobiose, α- and β-methylglucosides (13), glucose and xylose, to determine the route of breakdown of these carbohydrate components of their natural substrates, and, in addition, to investigate the relation of inorganic and organic phosphorus sources to mono- and disaccharide utilization.

Experimental

The media used for the carbohydrate studies, Part I, consist of the following:

Carbon source:

Glucose..20 g.

Or other carbon source as noted.

[1] Communication No. 180. This work was carried out under the auspices of the Office of Naval Research. Abridged from a part of the author's dissertation presented to the Graduate Faculty of Fordham University, 1949, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Supplement:

KH_2PO_4	1.5 g.
Neopeptone	1 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
Thiamine hydrochloride	2 mg.

Made up to 1 liter with tap water.

In Part II, in the studies of the influence of organic and/or inorganic phosphorus donors on mono- and disaccharide utilization by these molds, cellobiose (CB) and glucose (G), with adenosine triphosphoric acid (ATP), adenylic acid (AA), and KH_2PO_4 (P), were chosen. These donors were supplied to the media either alone or in combination. The experiments were so designed that the total phosphate was approximately equal in all cases, so that the metabolism exhibited would depend on the utilization of the particular phosphate linkage involved.

The media consisted of the following:

Glucose or cellobiose	10 g.
Neopeptone	1g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
Thiamine hydrochloride	2 mg.

To these materials were added the various phosphate sources, as follows:

In ATP media, 0.1 g. of adenosine triphosphoric acid was added/l., corresponding to 0.0146 mg. total P/ml.

In ATP-P media, 0.05 g. of adenosine triphosphoric acid (corresponding to 0.0073 mg. P/ml.) and 0.03 g. of KH_2PO_4 (corresponding to 0.0068 mg. P/ml.) were added/l., corresponding to 0.0141 mg. total P/ml.

In AA media, 0.15 g. of adenylic acid was added/l., corresponding to 0.0134 mg. total P/ml.

In AA-P media, 0.075 g. of adenylic acid (corresponding to 0.0067 mg. P/ml.) and 0.03 g. KH_2PO_4 (corresponding to 0.0068 mg. P/ml.) were added/l., making 0.0135 mg. total P/ml.

In P media, 0.06 g. of KH_2PO_4, corresponding to 0.0137 mg. total P/ml., was added/l.

Each of the above media was made up to 1 l. with tap water.

The components of the media were steam sterilized at 15–20 lbs. pressure for 20–30 min. before inoculation, with the exception of the organic phosphates, cellobiose (where noted) and the methyl-glucosides. These were found to be partially hydrolyzed by autoclaving, so they were dissolved as concentrates in distilled water and Seitz filtered. The sterile solutions were then added by means of a graduated sterile syringe in the quantities required for the individual flasks which already contained the autoclaved residual medium. This method proved satisfactory. Analyses of blanks showed that cellobiose was not split by this treatment, while inorganic phosphate formed about 1% of the total phosphate in the AA supplemented medium, and about 19% in the ATP medium. The latter figure indicates a tendency to spontaneous hydrolysis of the ATP in the slightly acid medium, but did not rise beyond this initial value in later analyses of the blanks.

Analytical

Methods used here were as in the previous communication, with the additions below.

Reducing sugars have been determined by the Munson-Walker method. In the case of glucose the standard tables were used for the relation of sugar to Cu_2O precipitated (2). For xylose the tables of Wise *et al.* were utilized (19).

For the determination of cellobiose the Munson-Walker technique was also applied. To obtain reducing values for cellobiose under the conditions of this analysis, determinations were carried out on known weights of cellobiose. The sample was prepared by the use of weighed quantities of the pure sugar (dried under vacuum for 48 hr.) made up to a known volume in distilled water, and portions of this solution were used in the analysis. The reducing values of known weights of cellobiose are shown in Table I. By this method the straight-line curve shown in Fig. 1 was obtained.

For glucose we obtained the reducing values shown in Table I, and found that they checked well with the standard tables. These values, and those of the literature, fall on a slightly curved line, with a transition point at approximately 110 mg. of glucose,

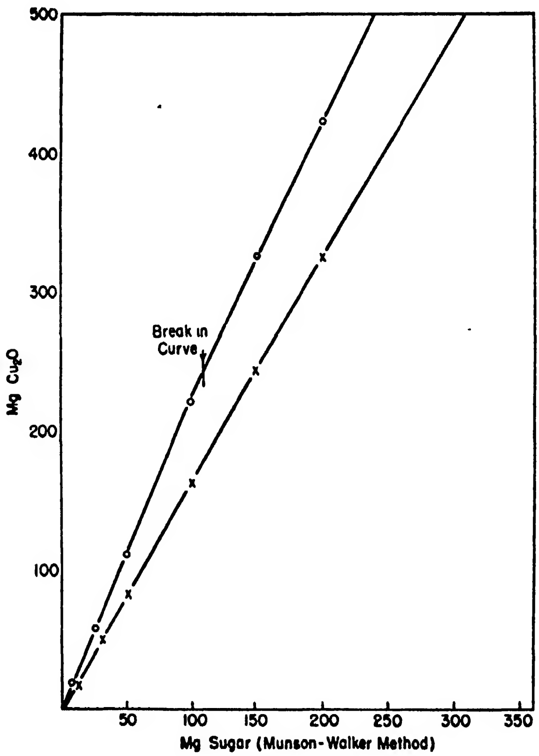


Fig. 1. Reducing values of sugars in Munson-Walker method.
×————× Cellobiose; ○————○ Glucose.

so we accommodated the points by two straight lines of best fit, one above and one below 110 mg. glucose, as shown in Fig. 1. A large graph was used for the plotting of the curves, so that, in practice, estimation to about 0.5 mg. was possible.

TABLE I

Reducing Values of Cellobiose and Glucose, Munson-Walker Method (Determined Experimentally)

Mg. sugar	Mg. Cu_2O formed by reduction Cellobiose	 Glucose
10	17.3, 17.4	19.5
25		57.4
30	49.8	
50	82.8, 82.5, 83.3	111.8, 111.6
100	163.9, 164.3, 163.8	221.7
150	244.6, 244.4	325.7
200	325.5	423.6

It was found that, by this method of approximation, in samples containing mixtures of the two sugars, the total reducing power of the mixture was equal to the sum of the reducing powers when each was determined individually. Table II shows the comparison of values calculated from the figure and those determined experimentally by Munson-Walker analyses.

TABLE II

Comparison of Graph-Calculated Values and Experimentally Determined Values, for Reducing Power of Cellobiose-Glucose Mixtures

Mixture		Cu_2O equivalent, graph			Cu_2O determined experimentally
Cellobiose	Glucose	Cellobiose	Glucose	Total	
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
50	0	83	0	83	82.9
40	10	66	23	89	88.6
30	20	50	45	95	95.6
25	25	42	56	98	98.0
20	30	33	67	100	99.8
10	40	16.5	89	105.5	105.4
0	50	0	111	111	111.1

From the graph curves it was also possible to calculate the relation of the sugars to Cu_2O precipitated, by deriving an equation for the curves. CB = cellobiose, G = glucose.

If x = mg. sugar, and y = mg. Cu_2O, then for CB

$$\begin{aligned} \text{When } x_1 &= 50,\ y_1 = 83, \\ x_2 &= 100,\ y_2 = 164, \end{aligned} \qquad \text{then } (y - y_1) = \frac{(y_2 - y_1)(x - x_1)}{(x_2 - x_1)},$$

$$(y - 83) = \frac{(164 - 83)(x - 50)}{(100 - 50)}, \qquad \text{Eq. 1}$$

$$y = 1.62x + 2.$$

That is, the wieght of cuprous oxide formed is approximately 1.62 × the weight of cellobiose + 2.

For G (since in practice, the weight of glucose formed from CB always fell below 110 mg., only this portion of the curve will be calculated):

$$\begin{aligned} \text{When } x_1 &= 50,\ y_1 = 112, \\ x_2 &= 100,\ y_2 = 222, \end{aligned} \qquad (y - 112) = \frac{(222 - 112)(x - 50)}{(100 - 50)}, \qquad \text{Eq. 2}$$

$$y = 2.2x + 2.$$

That is, the weight of Cu_2O formed is approximately 2.2 × the weight of glucose + 2.

Since hydrolysis of 1 g. of cellobiose forms 1.05 g. of glucose, it should be possible, knowing the reducing values of a solution of the sugar before and after hydrolysis, to calculate back to the amounts of the two sugars present in the mixture. The cellobiose was hydrolyzed to glucose (without destruction of glucose) by addition of an equal volume of 4.5 N H_2SO_4 to a sample, and heating for 2 hr. in a boiling water bath. The sample was then cooled, neutralized, and diluted to 50 ml. for use in the Munson-Walker analysis. To derive equations for the use of the hydrolysis method, Eq. 1 and 2 were utilized, by substitution, for reducing values before and after hydrolysis.

$$\begin{aligned} \text{Let } a &= \text{mg. CB in original solution,} \\ b &= \text{mg. G in original solution,} \\ 1.05a &= \text{mg. G formed from CB by hydrolysis,} \\ A &= \text{mg. } Cu_2O \text{ formed by original solution,} \\ B &= \text{mg. } Cu_2O \text{ formed by hydrolyzed solution.} \end{aligned}$$

Then

$$\begin{aligned} (1.62a + 2) + (2.2b + 2) &= A \quad \text{Before hydrolysis,} \\ (2.2[1.05a] + 2) + (2.2b + 2) &= B \quad \text{after hydrolysis.} \end{aligned}$$

Subtracting

$$\begin{aligned} (2.2[1.05a] + 2) + (2.2b + 2) &= B \\ (-1.62a - 2) - (2.2b + 2) &= -A \\ \hline 2.2(1.05a) - 1.62a &= B - A, \\ a &= \frac{B - A}{0.69}, \qquad \text{Eq. 3} \end{aligned}$$

and

$$(1.62a + 2) + (2.2b + 2) = A$$

$$b = \frac{A - 1.62a - 4}{2.2}. \qquad \text{Eq. 4}$$

The experimentally determined values for A and B, that is, the Cu_2O formed by a sample before and after hydrolysis, may be substituted in Eqs. 3 and 4, and by this means the relative proportions by weight of cellobiose and glucose present in a mix-

ture may be determined. This method has proved to be a very useful approximation (1–2% error) for the purposes of this study.

Inorganic phosphate values were determined on the filtered media by means of the Lowry and Lopez modification of the Fiske and SubbaRow method, a technique designed to give true values for inorganic phosphate since it should not hydrolyze the labile phosphate esters (9; 4a,b). While Kalckar *et al.* have pointed out that even these reagents will hydrolyze extremely labile esters, such as desoxyribosephosphate ester (5), Lowry and Lopez have shown that, using their method, it is possible to obtain true values for inorganic phosphate in the presence of acetyl phosphate and ribose-1-phosphate, which are normally regarded as unstable phosphates. Since this study is a comparative one, interested in general trends of the inorganic phosphate utilization rather than a fractionation of the organic phosphates, we believe that this method of analysis was satisfactory for our study.

I. Degradation of Carbohydrates

Merulius lacrymans

As shown in the previous paper, Melac, when grown on a glucose medium at 22°C., produces pyruvate (7), ethyl alcohol, acetate and oxalate from the sugar. Oxalate is present in largest quantity, its level paralleling the rate of growth. It was also possible to isolate glyoxylic acid as the 2,4-dinitro-phenylhydrazone from a glucose medium on which Melac grew at room temperature (up to 28°C.). With this mold, oxalate is the end-product corresponding to CO_2 in some organisms, and is apparently utilized only when glucose levels are low.

Comparative series were next set up on 3 cellulose sources, namely, filter paper (Whatman No. 2, about 1.5 g. per flask), and two commercial cellulose products, Solka-Floc, a wood powder, and Chemcot, a chemical cotton, in equivalent amounts. The cellulose was utilized as carbon source, and the basal medium supplement of salts, neopeptone and thiamine were added to it. Growth here was not at optimal rates, as compared with glucose, but all 3 sources were utilized, the filter paper being completely disintegrated in advanced growth. Mycelial weights could not be determined with any accuracy due to enmeshing of cellulose fibers with the mycelium. Inspection of Table III indicates that the filter paper cellulose is a slightly more favorable medium for growth, since oxalate levels rise more rapidly. Acetic acid and oxalate are the products of metabolism on all 3 sources, and both are further utilized. Traces of reducing sugars may be shown at intervals on all 3 products. The pH of the medium drops steadily during growth of this mold on cellulose, reaching about 3.3 in this series.

A phenylhydrazine derivative of the medium, m.p. 198°C., was formed from a 2-month culture at a period when traces of reducing sugar appeared in the Munson-Walker determination. Microscopic examination indicated that the crystals were those of cellobiose osazone (6). No glucosazone crystals were observable in this sample.

TABLE III

Metabolism of Merulius lacrymans on Cellulose Media

F = Whatman No. 2 filter paper; SF = Solka-Floc (Wood cellulose powder); CC = Chemcot (chemical cotton).

Age	Mycelial weight[a] mg./100 ml.	Reducing sugars	Acetate mg./100 ml.			Oxalate mg./100 ml.			pH
	F	F	F	SF	CC	F	SF	CC	
days									
Blank	—	None	—	—	—	—	—	—	6.01
28	26.8	Trace	None	None	None	15.0	None	4.5	3.96
50	45.7	None	6.0	Trace	5.1	15.2	3.9	3.5	3.32
65	45.3	None	None	None	3.9	3.0	20.9	19.6	3.36
78	50.6	None	None	None	None	6.2	1.9	5.8	3.67

[a] Approximate only (see text).

When the molds were first set up on an autoclaved regular cellobiose medium, early growth was irregular, and it was found that steam autoclaving was breaking down varying amounts of cellobiose (up to about 30%) by hydrolysis to glucose. As was later seen, this could make a profound difference in the response of the mold to the cellobiose medium. However, certain information was obtained which has undergone no change with later findings.

When Melac is grown on cellobiose medium, as shown in Table IV, the reducing power of the medium rose sharply during the early growth period, indicating that the disaccharide was split to glucose. Ethyl alcohol was also formed in amounts similar to those on glucose media, as well as oxalate, with the exception of the plateau period (65th day) when, despite disappearance of sugar, only a slight increase in mycelial

TABLE IV

Metabolism of Merulius lacrymans on Regular 1% Cellobiose Medium

Age	Mycelial weight mg./100 ml.	Reducing power, mg. Cu_2O/10 ml.	Alcohol mg./100 ml.	Acetate mg./100 ml.	Oxalate mg./100 ml.	pH
days						
Blank	—	155	—	—	—	5.93
17	32.2	160	Trace	5.3	None	4.28
32	61.6	147	6.3	47.0	None	3.43
51	203.8	125	1.4	4.0	71.6	2.42
65	221.4	45	1.4	·37.0	251.5	2.18
79	186.0	Trace	None	2.0	122.9	2.30
97	142.1	Trace	3.7	None	70.0	2.44

weight was shown, but an unusually large conversion into oxalate and acetate. In general, acetate was present only in small amounts, with this exception, and during the early growth period. The pH of the medium dropped steadily during growth, falling close to 2 at the peak of acid production.

Improved analyses of the autoclaved media (see anal. section), in which both cellobiose and glucose were present, as recorded in Table V, confirm that a rapid hydrolysis of cellobiose to glucose by Melac takes place, this process being so much faster than utilization, that ultimately all the sugar in the medium has been converted to glucose.

TABLE V

Cellobiose and Glucose Levels in Regular 1% Cellobiose Medium during Growth

CB = Cellobiose; G = Glucose

Age	Mycelial weight mg./100 ml.		Mg. sugar/10 ml. medium					
	Merulius larrymans	*Marasmius chordalis*	*Merulius larrymans*			*Marasmius chordalis*		
			CB	G	Total	CB	G	Total
days								
Blank	—	—	66.2	21.4	87.6	66.2	21.4	87.6
33	35.8	97.0	53.0	30.7	83.7	53.4	12.5	65.9
48	61.1	204.7	None	81	81	37.2	5.2	42.4
64	146.6	191.3	None	66	66	17	None	17
75	190.1	232.1	None	11	11	2	None	2

These autoclaved regular cellobiose media contained inorganic phosphate as phosphorus source. Later work has shown that this form of phosphate is unsatisfactory when pure cellobiose alone is present in the medium, but that, in the presence of glucose formed during autoclaving, it could be utilized. The effect of phosphorus source as related to carbohydrate utilization will be further discussed in the section on phosphorus utilization.

To study further the action of the molds on the glucoside linkage, α- and β-methylglucosides were used as substrates (18). Melac inoculation on β-methylglucoside medium produced traces of reducing sugar on a 13-day and a 1.5-month sample, while on α-methylglucoside sugar analyses were consistently negative. While this method is not aboslute for the detection of a glucosidase (since traces of glucose do not appear consistently, due probably to its rapid utilization for growth purposes), the results support our finding of the presence of β-glucosidase in Melac, and do not indicate the presence of an α-glucosidase.

(It may be noted in passing, however, that Ploetz, with press juice of Melac, showed the presence of maltase (14), which theoretically might be capable of splitting the α-methylglucoside.)

When Melac grows on xylose, the comparison with glucose indicates, that, while growth proceeds at a slower rate, ultimately the mold reaches a maximum mycelial weight which is as good as, or better than, that attained on glucose. Observation of several of these series indicate that as compared with glucose, the pentose is utilized quite sparingly from the standpoint of time and rate of disappearance, pointing to the utilization of intermediates. As may be seen in Table VI, oxalate accumulates to a somewhat higher level on this medium than on glucose. Alcohol is not produced in as large quantity, however, for it is frequently

TABLE VI

Metabolism of Merulius lacrymans on 2% Xylose Medium

Age	Mycelial weight mg./100 ml.	Residual xylose g./100 ml.	Alcohol mg./100 ml.	Acetate mg./100 ml.	Oxalate mg./100 ml.	pH
days						
Blank	—	1.88	—	—	—	5.19
18	37.6	1.90[a]	None	Trace	None	5.14
56	204.3	1.49	None	None	110.7	2.62
74	339.7	0.91	4.0	None	119.8	2.39
88	357.7	0.39	Trace	None	165.9	2.20
107	321.1	0.11	4.2	2.4	179.5	2.32
123	333.8	Trace	None	None	—	2.88

[a] Evidence of the presence of interfering reducing substances.

not present at all, while, on glucose it appeared consistently, although in small amounts. This may be accounted for by assuming that less alcohol is formed by the utilization of pentose.

Marasmius chordalis

As has been previously shown, Machor when grown on regular glucose medium at optimal temperature formed ethyl alcohol as the main isolable end-product of metabolism. This product accumulates during the early growth period (40–60 days), but when the logarithmic stage of growth is reached, the alcohol is rapidly dehydrogenated, and acetate and oxalate appear. These products are also further utilized. Here again

TABLE VII

Metabolism of Marasmius chordalis on Cellulose Medium
(Cellulose = Whatman No. 2 filter paper)

Age	Reducing sugar	Alcohol mg./100 ml.	Acetate mg./100 ml.	Oxalate mg./100 ml.	pH
days					
Blank	None	—	—	—	6.08
35	None	4.0	None	1.1	—
60	Trace	2.0	None	6.9	6.28
79	None	9.0	None	Trace	6.08
95	None	None	1.6	—	5.48
179	Trace	1.8	None	None	5.71

as with Melac, an inverse quantitative relationship is noted between ethyl alcohol and its dehydrogenation products acetate and oxalate. A strong test for pyruvic acid is also shown in early growth stages (7).

As shown in Table VII, Machor grown on filter paper cellulose shows a different picture from Melac. As on a glucose medium alcohol is the major end-product, while acetate and oxalate accumulate in relatively smaller amounts. Reducing sugars appear at intervals during growth. Alcohol, oxalate and acetate appear to be further utilized for growth, and pH changes are smaller than with Melac, from 6.08 down to 5.48 in this series.

On autoclaved cellobiose medium, as shown in Table VIII, no increase in reducing power of the medium could be observed. If glucose

TABLE VIII

Metabolism of Marasmius chordalis on Regular 1% Cellobiose Medium (Autoclaved)

Age	Mycelial weight mg./100 ml.	Reducing power mg. Cu_2O/10 ml.	Alcohol mg./100 ml.	Acetate mg./100 ml.	Oxalate mg./100 ml.	pH
days						
Blank	—	162	—	—	—	5.93
17	33.0	156	2.1	1.4	None	5.76
32	49.6	149	26.4	2.0	Trace	5.21
51	248.0	58	52.5	4.2	None	4.39
65	350.2	13	22.9	4.3	None	4.45
79	320.7	Trace	None	38.5	12.5	4.86
97	398.6	Trace	0.5	None	None	5.27

were formed from cellobiose prior to further degradation, either utilization was so rapid that the accumulation was not sufficient to be detected by the Munson-Walker analysis, or cellobiose was utilized directly. On this medium alcohol also accumulated, but the level remained high for a somewhat longer period than was observed in glucose media. Periodic sugar analyses indicated no glucose formation, and, in the autoclaved medium, where both cellobiose and glucose were present, both sugars appeared to be utilized at about the same rate.

For futher information as to the glucosidases of Machor, the organism was set up on media containing 0.5% α- and β-methylglucosides as carbon source (18). Machor showed about 4 mg. of glucose present in a 1-month sample on α-methylglucoside medium; all other samples were negative. This information supports the probability that this mold does not hydrolyze the cellobiose by means of a β-glucosidase, but rather ferments it directly.

TABLE IX

Metabolism of Marasmius chordalis on 2% Xylose Medium

Age	Mycelial weight mg./100 ml.	Residual xylose g./100 ml.	Alcohol mg./100 ml.	Acetate mg./100 ml.	Oxalate mg./100 ml.	pH
days						
Blank	—	1.80	—	—	—	5.19
46	147.7	1.83[a]	7.3	1.6	None	5.08
75	187.2	1.55	1.2	None	None	4.71
88	394.1	1.23	1.4	None	21.2	4.28
102	606.5	0.82	None	None	None	3.93
117	675.0	0.76	Trace	Trace	None	4.04
130	700.6	0.49	1.8	None	None	4.19

[a] Evidence for the presence of interfering reducing substances.

On a xylose medium, Machor, like Melac, but in an even more pronounced way, shows slow growth, which, however, ultimately reaches a maximum similar to glucose. From the data recorded in Table IX, we find the xylose is being used sparingly, and again alcohol accumulation is very slow, particularly striking here since this is the main isolable end-product of glucose utilization. The oxalate formed by Machor is normally utilized almost immediately with the one exception that, on the 88th day, after a period of very rapid growth there was

accumulation. Apparently the rate of carbohydrate breakdown had exceeded the rate of utilization of the intermediates.

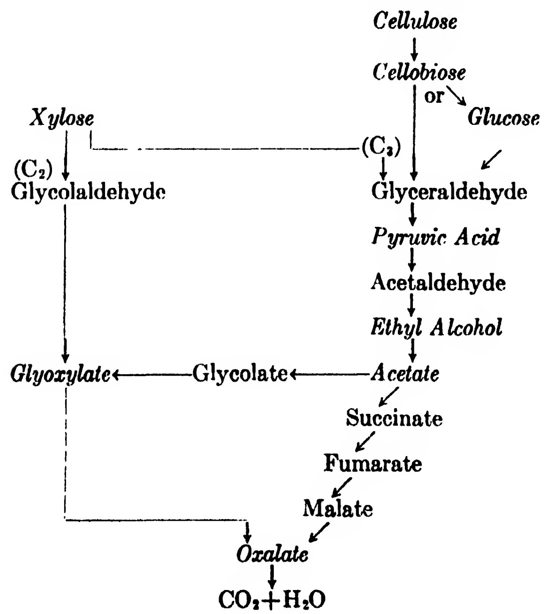
Ethyl alcohol medium is well utilized by Machor for growth, indicating that this is one of the reasons for its very low accumulation on xylose medium. However, since it is able to accumulate to high levels on glucose, despite this availability for utilization, a lesser formation must be the main reason for the low level in xylose media.

Discussion

Correlation of the experimental results on carbohydrate and intermediate metabolism studies obtained here and in earlier work from this laboratory (11c), make possible the postulation of a general scheme of hexose and pentose degradation in the cellulose-destroying molds as indicated in Scheme I. The products italicized in this scheme are those which either have been utilized as substrates, or have had their presence determined analytically, or both, during the course of this work. The sugar-splitting at this point is considered as a simple hydrolysis process, consideration of phosphorylation mechanisms being left for Part II of this paper.

SCHEME I

Scheme of Carbohydrate Degradation by Cellulose-Decomposing Molds



As far as the rate of attack on cellulose is concerned, it would appear from our recognition of cellobiose as the reducing sugar in a cellulose medium being attacked by Melac, that the enzyme cellulase, capable of breaking the cellulose linkages, functioned at a faster rate than the cellobiase of the mold (although we have shown the β-cellobiase also acts rapidly). That this is true is supported by the extremely rapid rate of even physical destruction of the cellulosic material. Under our conditions, the filter paper identity was completely lost by its becoming a suspended powder in the medium after prolonged attack, and in wood, a "de-cellulosed" wood skeleton is left after the mold attack. In addition, figures on spruce wood rotted by Melac, and cited by Falck, indicate that there is a sharp decrease in cellulose content and a definite increase in the copper number of the cellulosic material (3). Boswell has also shown that the early stages of attack by this mold on pine wood are hydrolytic and extremely rapid (1). He, however, was uncertain as to the size of the final product of this attack, suggesting that it is no smaller than a trisaccharide. Since we have obtained cellobiose as the main product of the attack on filter paper cellulose, it is quite likely that this would also be the main polysaccharide formed in wood attack. In the case of Machor, the filter paper cellulose is not disintegrated, and the mycelium grows on the surface of the paper, rather than completely penetrating the fibers.

The interesting variation in the degradation of glucosides by the two molds indicates a difference in their enzymatic potentiality. This is also displayed in their intermediate metabolism. Melac degrades cellobiose and β-methylglucoside through a preliminary hydrolysis to glucose, accomplished by β-glucosidase, while α-methylglucoside does not appear to be hydrolyzed. Machor, on the other hand, attacks cellobiose and β-methylglucoside directly, while α-methylglucoside is hydrolyzed. This observation is corroborated both by the sugar analyses on these media, and by the more rapid accumulation of the intermediates (notably oxalate with Melac and alcohol with Machor) on cellobiose medium as compared with glucose, despite the fact that, in the latter case, growth is slower.

The second variation, that of quantitative relation of secondary products, is displayed on the C_2 level. On glucose medium Melac accumulates large amounts of oxalate, but little alcohol; Machor accumulates alcohol, but little oxalate; both accumulate some acetate at intervals. The occurrence of all these products, although in different balances, would indicate that the same general route of metabolism is followed by these organisms, but that their enzymatic equipment is such that certain substances accumulate only at intervals when changes in the rate of metabolism are occurring. Oxalate may be considered as the end-product of sugar metabolism for Melac, being further used only in periods of extremely rapid growth, or when sugar levels are

low, *i.e.*, where the demand for carbon source is excessive. The high oxidation level of this product indicates that there is little advantage to be gained by further degradation to CO_2. In Machor, however, the higher growth rate makes it utilize even this product for growth purposes.

The possibility of correlating the processes of hexose and pentose metabolism by these organisms is also indicated by the chart, and shows a similarity to the pathways of carbohydrate breakdown in yeasts and *Fusaria* (11a). The proposed pentose scheme is logical according to our results. On a xylose medium the greater relative accumulation of oxalate with Melac, and the smaller accumulation of alcohol with Machor, as compared with that on glucose medium may be explained thus: oxalate is derived from the C_2 fraction, while alcohol is derived from the C_3 fraction. Since only half as much of the C_3 fraction is available from pentose, the formation of alcohol is also equally limited. In addition, the C_2 product formed by pentose-splitting, and the oxalate derived from it, are more likely to accumulate due to two reasons; first, the C_2 compound, oxalate, is at a higher oxidation level than the C_3 compounds derived from hexose, and is, therefore, less likely to be further converted, and secondly, the C_3 compounds derived from hexose are more likely to be removed from subsequent steps of the presented scheme by being subjected to other uses, for example, in protein synthesis. The product balance of both molds growing on glucose and xylose media support the likelihood of such a mechanism.

The postulation of glycolaldehyde as an intermediate in this pentose degradation as the C_2 fraction caused by the dichotomic splitting, has not been proven in this work, but Munson-Walker analyses indicate early formation of reducing substances in xylose media, which could include this substance. It has also been identified among the products formed by the action of a maceration juice from a bottom yeast containing added glyoxylic acid, in a reaction in which glycolic and oxalic acids were formed in equivalent amounts. Glycolaldehyde may, therefore, be considered a normal metabolism product of fungal growth (16).

II. Phosphorus Metabolism

Merulius lacrymans

Following the growth curves for Melac on glucose media, Fig. 2, it may be seen that inorganic phosphate, P, offers the best overall growth,

while the ATP and AA-P media offer the next best. The two latter media are apparently more easily broken down to the inorganic form necessary for growth of this mold on glucose. Therefore, it is logical to assume that both forms are split to inorganic phosphate through the intermediate adenosine diphosphate.

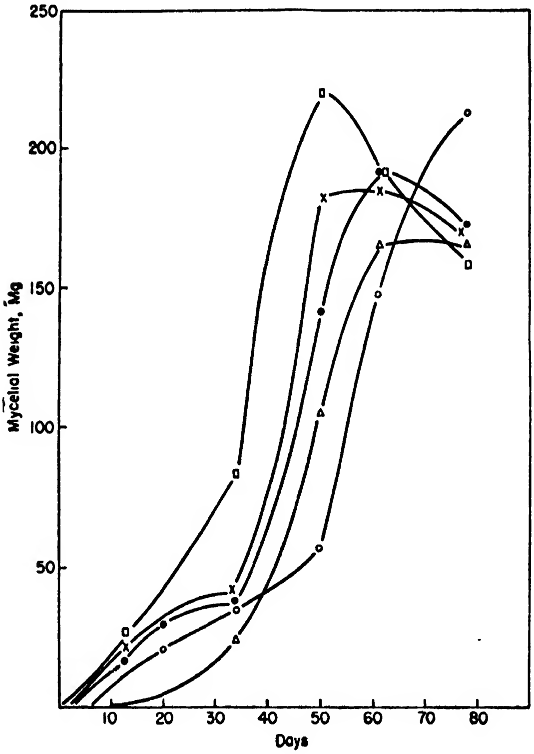


FIG. 2. Growth of *Merulius lacrymans* on 1% glucose medium with varying phosphate sources. Phosphate source: ×———× ATP; ○———○ ATP-P; △———△ AA; ●———● AA-P; □———□ P.

The steady and rapid lowering of the inorganic phosphate level in the P medium shown in Fig. 3, as compared with the levels in the organic phosphate media, confirms the fact that this is the preferred form during growth on glucose medium by Melac. It must be noted that, in the case of the organic phosphates, splitting to the inorganic form is almost complete before there is any evidence for utilization, and once this

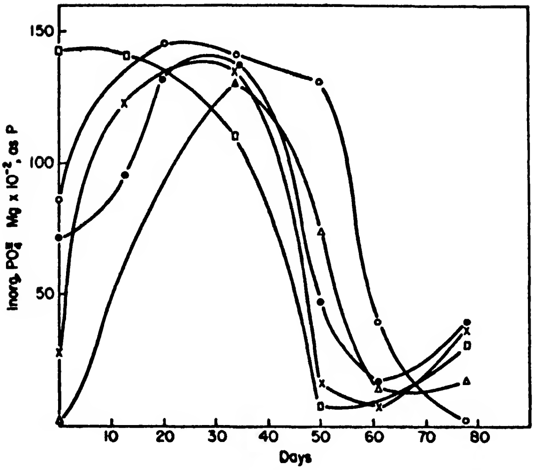


FIG. 3. Effect of varying phosphate source on inorganic phosphate levels in filtered medium from *Merulius lacrymans* on 1% glucose medium. Phosphate source: ×———× ATP; ○———○ ATP-P; △———△ AA; ●———● AA-P; □———□ P.

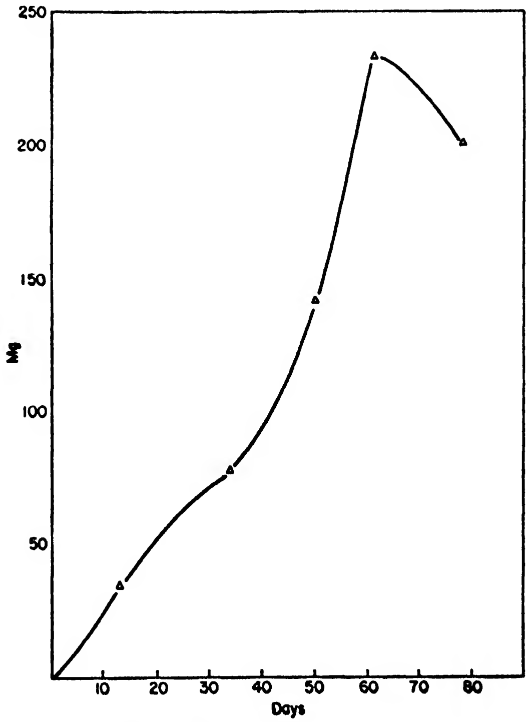


FIG. 4. Growth of *Merulius lacrymans* on 1% cellobiose medium. Phosphate source: △———△ AA. Ordinate = Mycelial Weight, Mg.

point is reached, inorganic phosphate disappears from the medium at a rate corresponding to the increase of mycelial weight.

In the case of Melac, growth on pure (Seitz-filtered) cellobiose medium, the situation is quite different (Figs. 4 and 5). Previous work had indicated that Melac hydrolyzes cellobiose to glucose during growth, and that it is also able to split β-methylglucoside to glucose, therefore functioning through β-glucosidase. Here, successful growth was obtained only in the case of the medium containing CB-AA as phosphate source. The growth on the CB-AA medium is superior to that on glucose, with the sole exception of the G-P medium, and ul-

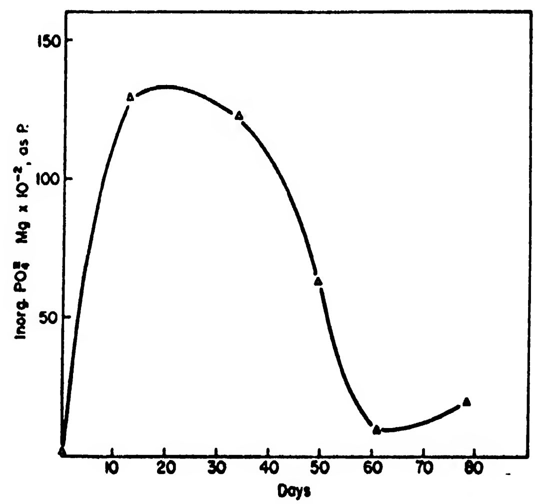


FIG. 5. Inorganic phosphate levels in filtered medium from *Merulius lacrymans* on 1% cellobiose medium. Phosphate source: △——————△ AA.

timately the maximum mycelial weight on the CB-AA medium exceeds even that on G-P. We can surmise here that the participation of the organic phosphate is significant in the hydrolysis of the disaccharide. The fact that AA is utilized in CB phosphorolysis in contrast to ATP may indicate that pyrophosphatase is not functioning in this system.

While, on the pure cellobiose medium used in this experiment, no growth was obtained with any of the phosphate sources except AA, as shown in Part I, growth could be obtained in all media of the autoclaved cellobiose medium in which both cellobiose and glucose were present. It is interesting to note, however, that, in both types of

media, the hydrolysis of cellobiose to glucose can be demonstrated analytically. Sugar analyses indicated that, in the P medium, the hydrolysis of cellobiose to glucose was considerably slowed down by comparison with the organic phosphate media.

Marasmius chordalis

With Machor, the picture is very different. With glucose, growth on the G-P medium is definitely the poorest, and we may thus conclude

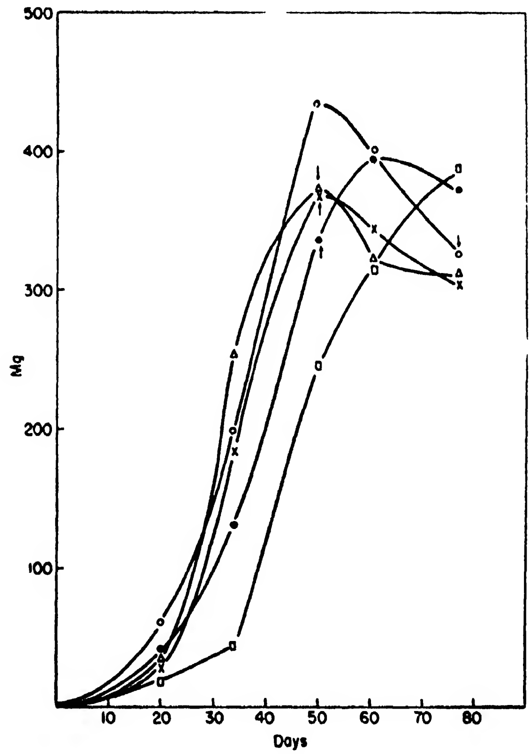


FIG. 6. Growth of *Marasmius chordalis* on 1% glucose medium with varying phosphate source. Phosphate source: ×——× ATP; ○——○ ATP-P; △——△ AA; ●——● AA-P; □——□ P. Ordinate = Mycelial Weight, Mg.

that the organic phosphates are the superior phosphorus donors in this case (Fig. 6). Growth in the presence of the organic phosphates is approximately equivalent, although the best overall growth source

appears to be the ATP-P combination. Here again we can observe by study of the inorganic phosphate levels, as recorded in Fig. 7, a very rapid splitting to inorganic phosphate; in fact, this hydrolysis has already reached its peak by the 20th day of growth. As with Melac the ATP-P is slowly split to the inorganic form. However, in *contrast* to Melac, this is the form of medium most useful in growth, again indicating that the organic form is significant here. This is further supported

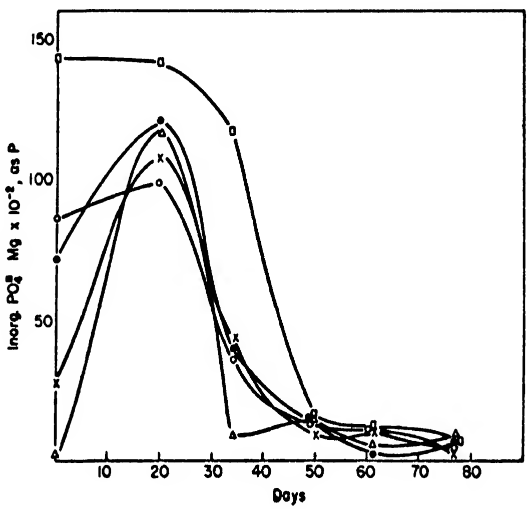


FIG. 7. Effect of varying phosphate source on inorganic phosphate levels in filtered medium from *Marasmius chordalis* on 1% glucose medium. Phosphate source: ×———× ATP; ○———○ ATP-P; △———△ AA; ●———● AA-P; □———□ P.

by the comparison with the growth curves and inorganic phosphate levels on the G-P medium, for it may be seen from these that the growth lags until a portion of the inorganic phosphate disappears from the medium. This lag period probably corresponds to a period of conversion of the P to an organic form, and only then will growth proceed to the logarithmic phase.

In the case of Machor on cellobiose medium, growth occurs on all of the media, but is much slower than on glucose, and a definite lag is shown before the onset of the logarithmic stage of growth (Fig. 8).

Previous work had shown that no glucose formation can be demonstrated during the growth of Machor on either cellobiose or β-methylglucoside, indicating that fermentation is direct. Observation of the inorganic phosphorus levels, as shown in Fig. 9, does not indicate that this lag phenomenon is attributable to any relation to phosphate. It

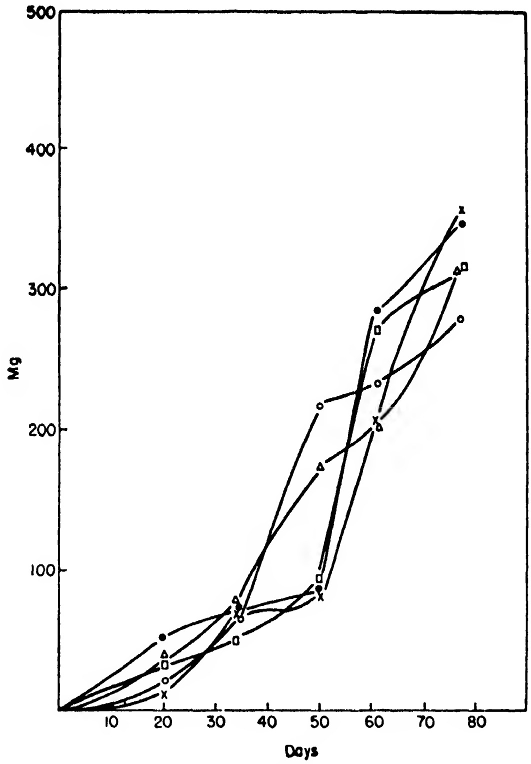


FIG. 8. Growth of *Marasmius chordalis* on 1% cellobiose medium with varying phosphate source. Phosphate source: X———X ATP; O———O ATP-P; △———△ AA; ●———● AA-P; □———□ P. Ordinate = Mycelial Weight, Mg.

may be due, however, to the direct utilization of cellobiose, without preliminary hydrolysis to glucose, a process which would be slower, due to the non-participation of phosphate. Ultimately, a stage is reached when some of the cellobiose has broken down to intermediates, and at which much of the split inorganic phosphate has been reincorporated into organic form. At this point the newly synthesized

organic phosphate may be functioning in the utilization of C_3 or C_2 intermediates, leading to their utilization for growth at the same rate at which they are utilized on a glucose medium.

To study the possible role of phosphorus at the C_2 site of metabolism, Melac and Machor were set up on a 0.5% ethyl alcohol medium, with ATP, AA and P phosphate sources in the same concentrations as used for the carbohydrate studies. Growth of both molds was slow on this

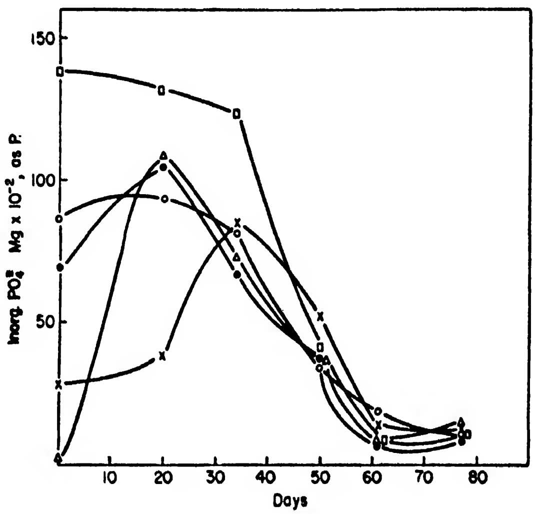


FIG. 9. Effect of varying phosphate source on inorganic phosphate levels in filtered medium from *Marasmius chordalis* on 1% cellobiose medium. Phosphate source: ×——× ATP; ○——○ ATP-P; △——△ AA; ●——● AA-P; □——□ P.

medium, Melac in particular showing slight growth, and then tapering off. That this mold did dehydrogenate the alcohol could be shown however, by the fact that in microscopic examination of the mycelium from the EtOH-AA medium one could recognize the presence of the characteristic crystals of calcium oxalate embedded in the mycelial threads of the mold. As recorded in Table X, Series I, however, mycelial weights were low, and appeared about equivalent on all 3 phosphates, being, perhaps somewhat superior on inorganic. A slight increase in inorganic phosphate level can also be noted.

Machor, however, shows a definite variation in growth on the 3

phosphates, ATP and AA being superior to P, the latter growth being at less than half the rate of the two organic forms (Table X, Series II).

TABLE X

Metabolism on Ethyl Alcohol Medium

Age *days*	ATP		AA		P	
	Mycelial weight mg./100 ml.	Inorg. PO_4 mg./cc. as P	Mycelial weight	Inorg. PO_4	Mycelial weight	Inorg. PO_4
Series I: *Merulius lacrymans.*						
Blank	—	38	—	21	—	149
34	7.4	49	7.0	32	9.2	147
Series II: *Marasmius chordalis*						
Blank	—	38	—	21	—	149
34	17.2	59	18.0	117	9.8	135
53	39.2	82	24.4	148	17.8	148
65	96.2	38	67.6	112	29.6	149

Splitting to inorganic phosphate is shown in ATP and AA, being partial in the case of the former and complete in the case of the latter. The inorganic phosphate rise is followed by a sharp fall.

Discussion

Since the organic phosphates function as phosphate donors, it may be assumed that, when growth on organic phosphate media is superior to that on inorganic phosphate media, the effect is due to participation of the organic phosphate in a process involving phosphorylation. On the other hand, when growth is superior on inorganic phosphate, which disappears from the medium at a rate which parallels growth of mycelium, it may be assumed that the degradation of the carbon source is independent of phosphorylation. Using this assumption as basis, the two molds show differences in their utilization of the mono-and disaccharides which may be summarized as follows:

Merulius lacrymans	*Marasmius chordalis*
1. Growth on glucose is superior in media containing inorganic phosphate, while organic forms function proportionally to the rate at which they are split to inorganic. Therefore, phosphorylation of hexose utilizing the adenylic route does not seem to be the essential route of carbohydrate degradation.	1. Growth is poorest on G-P, indicating the necessity for the presence of the organic phosphate forms. It is, therefore, likely that hexose is phosphorylated here by the adenylic route.
2. On cellobiose and β-methylglucoside, glucose is apparently formed by hydrolysis; therefore, the mold functions *via* a β-glucosidase.	2. There is no evidence for glucose formation or accumulation on either cellobiose or β-methylglucoside media; therefore, degradation is probably direct involving no preliminary hydrolysis.
3. Organic phosphates are the best sources for growth on media containing cellobiose, indicating that they act as phosphate donors in the phosphorolysis of the disaccharide.	3. The rate of degradation of cellobiose cannot be related to the type of phosphate available; therefore, phosphate plays no direct role in the degradation of cellobiose.
4. Growth on glucose formed from cellobiose is more rapid than that on media containing added glucose. This suggests that a phosphorylated glucose was formed.	4. Growth on cellobiose is much slower than on glucose, possibly due to the nonoccurrence of the phase of phosphorylation in the course of disaccharide degradation, since it is utilized directly.
5. Inorganic phosphate levels reach a peak, then drop at rates proportional to mycelial weight increase. This would indicate simple incorporation of inorganic phosphate into cell material.	5. Inorganic phosphate reaches a peak and then undergoes a sharp decline, at a rate independent of the carbohydrate source (mono- or disaccharide) and of the mycelial weight. This indicates that phosphate is functioning in the phosphorylation of intermediates at the C_3 or C_2 stage, possibly in a newly synthesized organic form.
6. On ethyl alcohol media, conversion to oxalate may be demonstrated, but mycelial weights are low, regardless of the phosphate source. The phosphorylation mechanism is probably not significant here.	6. On ethyl alcohol, growth is superior on the organic phosphates; therefore, the adenylic route of phosphorylation may be functioning here. Inorganic phosphate levels rise, then fall sharply.

7. In general, it may be said that, for both molds, the ATP-P medium compares favorably with the other combinations of inorganic and organic phosphate when the overall picture is considered, that is, time of onset of growth, time of onset of the logarithmic phase of growth, and maximum mycelial weight attainment. The availability of both the organic and inorganic forms of phosphate in this medium is doubtless responsible for its favorable role.

It is instructive to compare the growth of Melac and Machor on cellobiose and glucose media with that of other molds on poly- and

monosaccharides. O'Connor, working with *Fusarium lini* Bolley, showed that maltose and lactose may be fermented directly and indirectly, and are not, in the case of direct fermentation, necessarily phosphorylated. In the case of the metabolism of trehalose also, she found direct fermentation (12). The present series of investigations have shown that Machor ferments cellobiose directly, and with no evidence of phosphorylation in this step.

As for the wood-destroying molds, Nord and Sciarini have indicated that when *Merulius niveus* is growing on glucose and raffinose, no transfer of phosphate to creatine (as acceptor) can be demonstrated, indicating that here phosphorylation is not an integral part of the carbohydrate degradation. *Merulius tremellosus* and *Merulius confluens* did not ferment raffinose to any measurable extent under their conditions (using inorganic phosphate) (11b). In our investigations with Melac, pure cellobiose was not split in the presence of inorganic phosphate alone as phosphorus source, but in the presence of the organic phosphate, AA, the hydrolysis to glucose proceeded successfully.

All of these observations support the general postulation that the intervention of phosphate (preferably organic) may be shown in some cases where the hydrolysis of the polysaccharide precedes further utilization, but, where direct fermentation is involved, phosphate may or may not enter into the process, depending on the organism involved.

Inspection of Figs. 3, 5, 7, and 9, will indicate that each mold presents a very definite pattern for inorganic phosphate utilization in the medium, such levels rising to a peak which represents a very large percentage of the total phosphate added. This peak is followed by a sharp decline in phosphate levels until the plateau period of growth, when equilibrium is attained. This drop in inorganic phosphate levels has been noted in other organisms and attributed to various reasons (11a, 10, 8a).

In the case of our observations with Melac, the rate of disappearance of inorganic phosphate from the medium follows closely the rate of cell formation, as evidenced by mycelial weight increase. It is likely here, as in *Fusaria*, that the main function of the inorganic phosphate lies in cell building, and that it is not directly involved in intermediate breakdown. That this is not in contradiction to energy considerations is pointed out by Lipmann (8b), who noted that thermodynamically the phosphate mechanism represents a restraint rather than a promotion of the pyruvate reaction, and that it is not surprising that certain organisms degrade pyruvate without phosphorylation, as indicated by

Stumpf, in enzyme preparations from fresh suspension of *Proteus vulgaris* (17).

On the other hand, rates of utilization of phosphate by Machor show an extremely sharp drop after the peak stage, and this decline follows a pattern which appears to be independent of growth and of carbohydrate source (*i.e.*, mono- or disaccharide). This would point to the likelihood that, in this organism, similarly to Lynen's observations on yeast, intermediary phosphorylation is involved in the carbohydrate metabolism at the C_3 or C_2 stage.

The results of the experiments on ethyl alcohol media support the previous postulations derived from inspection of phosphate levels. The very slight influence of the phosphate source in the growth of Melac on this medium, with the possible slight superiority of inorganic phosphate, indicate that the phosphate is not an integral factor at this stage of metabolism. The definite superiority of the organic over the inorganic phosphate in the case of Machor, indicate that, in this mold, in contrast to Melac, phosphorylation does enter into the intermediary metabolism. The slow rate of growth on inorganic phosphate indicates that this must be converted into the organic form for utilization.

These studies demonstrate the variation that can be observed experimentally on organisms closely related in nature, and exemplify the danger of postulating general routes which are supposed to be representative for a group of organisms or cells in general. While it sometimes appears practical to emphasize the desirability of a general theory to cover such mechanisms in carbohydrate degradation, it should not impede recognizing the individual differences in the behavior of a specific living organism.

Acknowledgments

The following items were obtained through the courtesy of individuals or groups:

Cultures. The *Merulius lacrymans* strain from Dr. Wm. J. Robbins of the New York Botanical Gardens, New York 58, N. Y.; the culture of *Marasmius chordalis* from Prof. E. Melin, Dept. of Physiol. Botany, Royal University, Uppsala, Sweden.

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Chemcot from the Southern Chemical Cotton Co., Chattanooga, Tenn.

Solka-Floc from the Brown Co., New York.

Summary

1. Growth of *Merulius lacrymans* and *Marasmius chordalis* on cellulose, glucose, cellobiose, α- and β-methylglucosides, xylose, and ethyl alcohol are compared. These studies have indicated that the two molds differ in the following respects:

Melac	Machor
(a). Causes intense physical destruction of filter paper cellulose.	(a). Does not disintegrate the filter paper during growth.
(b). Hydrolyzes cellobiose and β-methylglucoside before further utilization.	(b). Attacks cellobiose and β-methylglucoside directly, with no evidence for preliminary hydrolysis.
(c). α-Methylglucoside appears to be utilized without preliminary hydrolysis.	(c). α-Methylglucoside is hydrolyzed by this mold.
(d). Accumulation of intermediates and rate of growth on cellobiose are about equivalent to that on glucose, indicating that the metabolism is really that of glucose.	(d). Accumulation of intermediates in cellobiose degradation is higher than on glucose, supporting the view that cellobiose is utilized directly.
(e). In metabolism of cellulose, cellobiose and glucose, oxalate is the main isolable end-product of metabolism. Ethyl alcohol and acetate are isolated in smaller amounts.	(e). On cellulose, cellobiose and glucose media, ethyl alcohol is the main isolable end-product of degradation. Oxalate and acetate are obtained in smaller amounts.
(f). Grows poorly on ethyl alcohol medium.	(f). Grows well on ethyl alcohol medium.

2. A scheme is presented for carbohydrate degradation in the cellulose-decomposing molds, and supporting evidence is offered. The pentose degradation portion is indicated as a C_3–C_2 split by the lesser accumulation of the C_3 fraction derivative, ethyl alcohol, and the greater accumulation of a C_2 derivative, oxalic acid, in xylose metabolism, as compared with glucose metabolism.

3. Details of a method of analysis for cellobiose and glucose in the presence of each other have been worked out, and tables and a graph for the use of the Munson-Walker method with these sugars are presented.

4. Based on the comparison of growth rates on inorganic and organic phosphates, and on the assumption that organic phosphates can function as phosphorus donors, the following variations between the molds have been demonstrated:

Melac	Machor
(a). degrades cellobiose through a preliminary phosphorolysis to glucose; by this means a phosphorylated glucose is formed which is utilized more rapidly than added glucose.	(a). Since it has been shown that Machor degrades cellobiose without preliminary hydrolysis, and since no relation of phosphate levels to the rate of cellobiose degradation can be demonstrated, it is likely that no phosphorylation takes place in the direct fermentation of cellobiose.
(b). Melac does not utilize the phosphorylation route during the degradation of supplied glucose.	(b). Machor phosphorylates glucose.
(c). Melac does not degrade ethyl alcohol *via* the adenylic route.	(c). Machor utilizes phosphorus during conversion of ethyl alcohol.

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Lipase Determinations with the Aid of Polyvinyl Alcohol

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Introduction

Some of the *Fusaria* are characterized by the presence of fat-forming enzyme systems, the actions of which have recently been shown to be interrelated with pigment formation in some of these organisms (1, a, b). Studies on the mechanism of carbohydrate → fat conversion in these molds have led to an investigation of the enzyme system involved in the formation and degradation of neutral fats. Part of this problem required the identification of the enzyme present in *Fusarium lini* Bolley (FlB) as either an esterase or a true lipase. The purpose of this communication is to present the method developed and the results obtained during the course of this work.

A review of the literature reveals the recommendation of numerous procedures for the determination of esterase or lipase activity (2,3,4), the limitations of which have gradually come to the fore (5,6,7). As a result of these restricting conditions, variations in the manner of approach to the developed problems have been reported (6–11). However, it must be kept in mind, that, if restrictions do exist, often the failure is not due to the method employed, but to the complexity of the enzyme system to be studied: *i.e.*, the variations applied to the study of esterases and lipases usually do not conform to the actually prevailing conditions.

One of the basic difficulties in the proper study of lipolytic activity, and also in the establishment of the presence of a true lipase, is the emulsification of water-insoluble substrates, a fact realized as far back as 1906 (12). The inconveniences encountered in such systems finally led Archibald (8) to employ the "unnatural" water-soluble Tweens as substrates. On the other hand, to circumvent the problem of proper emulsification, some investigators have limited their studies to water-soluble or slightly water-soluble substances, whereas others, using natural water-insoluble substrates, have applied sodium glycocholate and sodium oleate (13,14), gum acacia (15,16), gum arabic (17), glycerol and bile (18), and bentonite (19) as emulsifying agents. The difficulties that arise with the use of such substances are: (a) non-homogeneous

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emulsions, (b) unstable emulsions, either in the preparative stages or during the actual incubation periods, and (c) difficulty in determining the end point when employing titrimetric procedures.

Nord *et al.* (20) were the first to refer to the use of polyvinyl alcohol (PVA) as an emulsifying agent and, in later work (21), employed the polymer as a stabilizing agent in the preparation of various emulsions for *in vivo* studies involving some enzyme systems of FlB. Contrary to its gold number, PVA had also proved to be far superior to gum arabic or gum tragacanth in catalytic work (22). Thus, it was thought advisable to extend the possible use of PVA to *in vitro* studies involving lipolytic action.

Experimental

Cultures Employed

As heretofore, our strain, FlB, No. 5140, was employed in these investigations. Stock cultures were maintained on the customary nutrient medium (23). The procedures for growing the mold, collecting the mats, drying and grinding them were the same as those employed in previous studies (24). The dried and finely-ground mycelium was then stored in a vacuum desiccator over conc. c.p. H_2SO_4 at $-$ 14°C. and used, as required, for the preparation of the enzyme material.

Preparation of PVA

Ten g. of PVA (medium viscosity, Type B) are stirred mechanically into 1 l. of distilled H_2O at room temperature for 30 min. At the end of this time, 5 ml. of 0.1 *N* HCl are added and the slurry heated at 75–85°C. until solution is complete (about 1 hr.). The mixture is heated one more hour, after adding 100 ml. of distilled H_2O. The solution is then cooled, filtered and brought to pH 7.0 with 0.1 *N* NaOH (about 5.5 ml. are required).

Preparation of Enzyme Material

Three g. of a concentrated lipase preparation obtained from the dry FlB mycelium are suspended in 100 ml. of distilled H_2O in a Knapp-Monarch blendor at *low* speed for 1 min., left to stand at room temperature for 15 min. and centrifuged for 30 min. at 2,000 R.P.M. The supernatant is then decanted and strained through two layers of muslin. This only slightly turbid preparation of lipase is then used in these determinations.

When it is desired to study the action of pancreatic lipase, a similar preparation can be obtained by treating commercial steapsin in like manner.

Preparation of Emulsions

The desired amount of ester (*M*/10) or oil is placed into 100 ml. of the PVA solution in a Waring blendor for 5 minutes. The emulsion is left to stand for a few minutes and then used.

Method of Determination

To 10 ml. of the PVA-emulsified substrate, contained in a 125 ml. Erlenmeyer flask, are added 5 ml. of buffer (McIlvaine, pH 7.0) and 5 ml. of the enzyme preparation. The mixture is shaken gently and incubated for 4 hr. at 37°C. with constant shaking. (The controls contained enzyme preparations that had been brought to a boil and cooled.)

At the end of the required time, 10 ml. of 1:1 alcohol-acetone solutions are added to terminate the enzyme action and to break up the emulsion. 0.25 ml. of a 1% alcoholic phenolphthalein solution is added and the whole allowed to stand for 10 min. The mixture is then titrated with 0.05 *N* alcoholic KOH.

All analyses were conducted in duplicate.

Results and Discussion

The results of these studies are recorded in Table I.

That FIB seems to contain a true lipase is evidenced by the fact that the triglycerides, *e.g.*, triacetin and tributyrin, are hydrolyzed at a

TABLE I

Action of FIB Lipase on Various Substrates

Substrate tested	Am't. added per 100 ml. PVA	Control	Sample	Difference
		(ml. of 0.05 *N* alcoholic KOH)		
PVA (alone)	None	7.20	7.30	0.10
Ethyl acetate	1 ml.	7.50	9.50	2.00
Ethyl propionate	1.1 ml.	7.75	9.70	1.95
Ethyl butyrate	1.3 ml.	7.35	9.00	1.65
n-Butyl butyrate	1.6 ml.	7.75	9.75	2.00
Ethyl laurate	2.2 ml.	7.80	8.50	0.70
Ethyl stearate	3.1 g.	7.75	8.10	0.35
Triacetin	1.9 ml.	7.50	12.15	4.65
Tributyrin	2.8 ml.	7.70	11.20	3.50
Olive oil	3.0 ml.	7.90	9.20	1.30
Fusarium oil[a]	3.0 ml.	11.75	13.25	1.50

[a] This oil was obtained from FIB (24). The entire amount of the semisolid oil was not emulsifiable, and, therefore, after treatment in the blendor, the emulsion was filtered before using.

faster rate than the simple esters. Also the oils are hydrolyzed faster than the higher simple esters.

Hydrolysis of p-Methoxy Methyl Cinnamate

In a study of the formation of *p*-methoxy methyl cinnamate (25), it was observed that this crystalline ester, formed during the growth of *Lentinus lepideus*, gradually disappeared from the medium. This finding indicated that certain enzymes, discharged into the medium during the slow disintegration of the cells, hydrolyzed the insoluble ester. In a later study (26), the formation of lipides in the same mold was demonstrated. This latter finding substantiates the presence of a lipase in this organism.

The above observations, plus the fact that lipases have been shown to be capable of hydrolyzing certain aromatic acid esters, prompted us to find out whether or not a concentrated mold lipase (*i.e.*, of FlB) would be capable of hydrolyzing the ester *in vitro*. Thus, by inference it would be possible to postulate the initial step in the disappearance of the ester, a compound of great significance in the mechanism of carbohydrate → lignin conversion (25).

TABLE II

Action of FlB Lipase on p-Methoxy Methyl Cinnamate

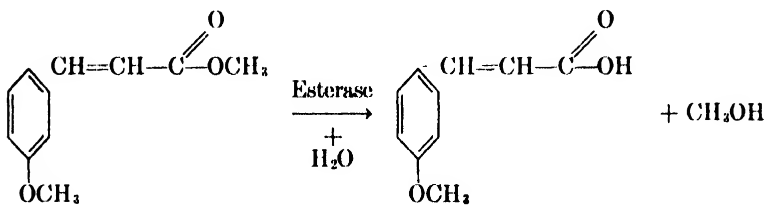
Experiment no.	Control	Sample	Difference
	(ml. of 0.05 *N* alcoholic KOH)		
1	7.50	8.50	1.00
2	8.35	9.80	1.45

Thus, in *two* separate experiments, the methods described above were applied with a few alterations. One of these was that a 7 hr. incubation period was employed.

The two experiments were: (1) The emulsion was prepared by treating 2 g. of the ester in 100 ml. of PVA as previously described and filtering the emulsion, since only a small quantity of the ester is emulsifiable. The rest of the procedure was the same. (2) Here the use of PVA was eliminated and, in place of 10 ml. of PVA-emulsified substrate, we used 100 mg. of the ester in solid form after adding 10 ml.

of distilled H_2O to bring the total volume to 20 ml. The rest of the procedure was again the same. The data are recorded in Table II.

Thus, the ester *is* hydrolyzed by the FIB lipase both in the solid form and when emulsified. By inference then, it can be stated that the first step in the transformation of the ester formed by the action of *Lentinus lepideus* on wood, glucose, xylose, *etc.*, is probably:



It should be mentioned that the same result was obtained with a preparation of commercial steapsin.

Comments

The evidence presented shows that PVA can be advantageously used as an emulsifying agent for water-insoluble substrates in the study of lipolytic activity. The emulsions produced are stable during the incubation periods involved, easy to pipette and handle, and can be diluted with buffer and enzyme material in a homogeneous way without disturbance. Another advantage is that they can be easily broken up with organic solvents. However, the disadvantages are that the end point of the titration in the procedure described is not sharp, that the error of the method amounts to $\pm$ 0.25 ml. of 0.05 N alcoholic KOH, and that substances such as trilaurin and tristearin cannot be emulsified in the concentrations desired ($M/10$).

It was, therefore, of interest to amplify the method in such a way that no difficulty would be experienced in calling the end point. It was not found necessary to extend any modification of the procedure to all the substrates previously employed, since: (1) it was desired to determine whether or not FIB possessed a true fungal lipase. This point was conclusively answered by the analytical data already presented. (2) The use of a single substrate (*e.g.*, olive oil) in the alternate procedure should give approximately the same results as the original method, using the same enzyme preparation.

For our purposes then, olive oil was chosen as the test substrate. All

preparative methods are the same as those described, and the only difference is in the actual determination.

Alternate Procedure

At the end of the required incubation time, 30 ml. of a 1:1 alcohol-acetone solution are added to stop the reaction and break up the emulsion. The whole is allowed to stand for 10 min. and filtered, using a fluted No. 2 Whatman paper. A 25 ml. aliquot of the *clear* filtrate is removed and 0.3 ml. of 1% alcoholic phenolphthalein are added. The solution is then titrated with 0.05 N aqueous NaOH. (Aqueous alkali is used to prevent precipitation of the PVA during the titration.) The titer thus obtained is multiplied by 2 and reported as such. Thus, it was found that a value of 1.50 is obtained by the altered method as compared to 1.30 as originally observed.

This alteration then presents *no* difficulty in observing the end point and substantiates the data initially presented.

Acknowledgment

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Summary

1. A method for the determination of lipolytic activity employing polyvinyl alcohol as the emulsifying agent for water-insoluble substrates is presented.
2. *Fusarium lini* Bolley contains a true lipase.
3. The lipase system of *Fusarium lini* Bolley (and of the pancreas) can hydrolyze p-methoxy methyl cinnamate either in an emulsified or solid form.

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On the Mechanism of Enzyme Action. XL. The Interaction of Solanione, Riboflavin, and Nicotinic Acid in the Carbohydrate→Fat Conversion by Certain *Fusaria*

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Introduction

It has been shown that the addition of solanione, a pigment obtained from *Fusarium solani D_2 purple* (FsD) (1), increased the desaturation of the fat formed by *Fusarium lini Bolley* (FlB) (a non-pigment producer), while its addition to a pigmented *Fusarium lycopersici* (Flyco) had no effect on the composition of the fat produced by the latter (2). A subsequent study with several naphthoquinones structurally related to solanione (S) has revealed thus far that these compounds can quantitatively affect fat formation in FlB (3).

Evidence has been presented that the genus *Fusarium* contains powerful dehydrogenating systems (4a,b), and that FlB, in particular, possesses dehydrogenases capable of acting on both saturated and unsaturated fatty acids (5). With the presence of such enzymes established, and knowing that riboflavin (R) and nicotinic acid (NA) can play an important role as co-enzymes in dehydrogenating systems, it was of interest to study their effects on fat formation in FlB.

Moreover, since Flyco could be obtained both pigmented (6) and unpigmented, it could serve as an ideal agent to demonstrate the interaction of these vitamins and mold pigments in the carbohydrate→ fat conversion.

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EXPERIMENTAL

Cultures Employed

The following *Fusaria* were used in these investigations: (1) FlB, No. 5140, obtained originally from the Biologische Reichsanstalt, Berlin-Dahlem, through the late Dr. H. Wollenweber; (2) Flyco, Strain No. R-5-6, obtained through the courtesy of Dr. S. P. Doolittle, U. S. Dept. Agriculture, Beltsville, Md. Stock cultures of the FlB were maintained on the usual nutrient medium (7). All Flyco stocks were maintained on potato dextrose-agar. Pigmented Flyco could be obtained by inoculating fresh isolates of the strain from tomato plants directly into potato dextrose-agar. Continued subculturing on any type of medium (including that mentioned) resulted in the loss of pigment-producing ability. The formation of the pigment could be discerned only when the respective cultures were grown on a Raulin-Thom medium. Previous work had indicated that varying cultural conditions have a marked effect on the pigmentation in Flyco (8).

Analytical Procedures

Growing of the Molds. The procedures for growing the *Fusaria*, collecting the fully grown mats and preparing them for subsequent investigations were the same as those employed in previous studies (9), applying additional precautions in the washing of the mats to insure complete removal of added substances. The S was dissolved in acetone, the R in a 1:1 mixture of acetone and water, and the NA in water, and these were added to the various media prior to sterilization.

Ten flasks were set up in each series of experiments and, at the end of the required time of incubations, the contents were combined for analysis.

Sugar, Fat and Sterol Determinations; Iodine Values. The filtrates were analyzed for sugar (10). The fat and sterol determinations were carried out as previously described (9). It should be pointed out that total lipide determinations were carried out by $CHCl_3$ extractions, whereas iodine values, spectrophotometric analyses and sterol contents were run on a petroleum ether (B. P. 30–60°C.) extract of the dry mycelium. These oils were obtained by drying the petroleum ether extract with anhydrous Na_2SO_4, filtering through a sintered glass funnel, and evaporating off the solvent *in vacuo* under a stream of nitrogen. Whenever the various fractions of the fats were collected for more than one day, they were stored in the cold under nitrogen.

The iodine values were determined according to the Hanus method (10).

Fat Coefficient. The fat coefficient or carbohydrate conversion factor is defined as the number of g. of total lipide produced/100 g. of carbohydrate utilized.

Riboflavin Content. The content of vitamin B_2 was measured on the dry, undefatted mycelium, employing essentially the method of Arnold (11) with a Pfaltz and Bauer fluorophotometer.

Spectrophotometric Analysis. The ultraviolet absorption curves of the various isolated fats were obtained with a Beckmann spectrophotometer, using isooctane of high purity as the solvent.

A. Experiments with FlB

For the purpose of these investigations, the FlB was grown in the dark on a Raulin-Thom medium containing 2.5% glucose.

During the course of certain enzyme isolation studies (to be reported later) with FlB grown under the same conditions, it was found that the supernatant obtained by alcoholic precipitation of aqueous suspensions of the mycelia possessed a deep yellow color and also a greenish yellow fluorescence. This was suspected to be due to riboflavin. The presence of this vitamin in *Fusaria* had been reported (12); however, the use of "isolates" in this study was of no help to us, since the particular species of *Fusaria* were not specified.

Identification of Riboflavin. Using the material referred to above, the final identification was based on combinations of several methods (13,14,15).

Nine liters of the alcoholic solution were concentrated *in vacuo* in the dark. After adding 1 ml. of conc. H_2SO_4, and autoclaving for 15 min. at 20 lbs. pressure, the mixture was cooled and brought to pH 4.5 with 30% NaOH. Two g. of Hyflo Super-Cel were added, and the whole stirred for 30 min. The filtered residue was treated with boiling water and refiltered. The combined filtrates were passed by gravity through a column of Florisil (18 cm. long and 2.5 cm. diam.) in the dark. The column had been previously washed with 100 ml. of 2% acetic acid followed by 50 ml. distilled H_2O. In this way, all the riboflavin was removed from the solution. However, the filtrate was still yellow. This is due to another colored substance that is not reduced by $Na_2S_2O_4$, not adsorbed by Florisil, not soluble in *n*-butyl alcohol or chloroform, and which is oxidized by $KMnO_4$ in acid solution (this substance could be removed prior to adsorption by treating the acidified solution with 4% $KMnO_4$). The column was then washed with hot distilled water until the washings were colorless and the flavin eluted with 100 ml. of 20% pyridine in 2% acetic acid. The eluate was then extracted with ethyl ether in a liquid-liquid extractor for 15 hr. The flavin solution was removed, and extracted 3 times with 100 ml. portions of *n*-butyl alcohol. The alcohol was completely removed *in vacuo* and the bright yellow residue dissolved in 15 ml. of hot H_2O and filtered. The identity of the compound as riboflavin was established by using this solution and running a comparative ultraviolet absorption curve with synthetic riboflavin in H_2O, measuring D/D max. *vs.* λ.

Quantitative determinations on the dry, undefatted FlB mycelium and on the culture filtrate showed the presence of 35 γ R/g. of mycelium and 0.05 γ R/ml. of culture filtrate.

Comparative Effects of Solanione and Riboflavin on Fat Formation in FlB

Since solanione can affect fat formation in FlB (2,8), and having established the presence of riboflavin, the question arose as to whether this vitamin might also interact in the genesis of fats. Consequently, for exploratory observations, FlB was grown with and without added S and R in the amounts indicated in Table I.

TABLE I

Comparative Effects of Solanione and Riboflavin on Fat Formation in FlB

Sample no.	Added substance	Mycelial wts.	Total lipide	Iodine value	Riboflavin γ/g. mycelium
	mg./l.	*g./l.*	*per cent*		
1	Control	3.88	9.17	110	31
2	1.0 S.	3.05	7.48	148	34
3	0.2 R.	3.38	7.00	150	34

S = Solanione; R = Riboflavin.

The results of the experiment are recorded in Table I. It will be noticed that the presence of the vitamin produced the same effects as S, *i.e.*, the I. V. is increased, indicating the synthesis of a more unsaturated fat. Neither compound affects the production of R in the mycelia. Detailed experiments were set up to determine whether: (1) the two compounds could produce the same effect by similar or different mechanisms; (2) varying amounts of added R would cause the formation of fats of varying unsaturation; (3) other compounds, which are capable of interacting with dehydrogenating systems (*e.g.*, NA) could bring about the same effect.

Effects of Riboflavin and Nicotinic Acid on Fat Formation in FlB

The results of this study are recorded in Table II and in Fig. 1. It can be seen that both vitamins decrease the percentage of total lipide and the fat coefficient, but raise the I. V. as compared to the control.

TABLE II

Effects of Riboflavin and Nicotinic Acid on Fat Formation in FlB

Sample no.	Added substance	Mycelial wts.	Total lipide	Sterol in isolated fat	Iodine value	Riboflavin γ/g. mycelium	Fat coefficient
	γ/l.	*g./l.*	*per cent*	*per cent*			
1	Control	5.50	8.55	7.3	111	30	1.88
2	40 R.	5.32	8.23	8.0	133	30	1.75
3	200 R.	5.52	6.46	8.0	131	30	1.43
4	800 R.	5.37	6.88	10.4	157	32	1.50
5	500 N. A.	5.30	7.01	9.5	153	29	1.49

R. = Riboflavin; N. A. = Nicotinic acid.

Neither compound affects the production of R. Furthermore, varying amounts of R cause the formation of fats of varying unsaturation.

In previous work from this laboratory (2) it was found that the spectrophotometric curves of the fats isolated from FlB grown with and without added S showed a significant deviation between 240 mμ and 265 mμ, indicating that a partial change in the composition of the fat had occurred. However, in the range 265-310 mμ, the two absorption curves were practically identical, denoting little change in the ergosterol content of the fats (16).

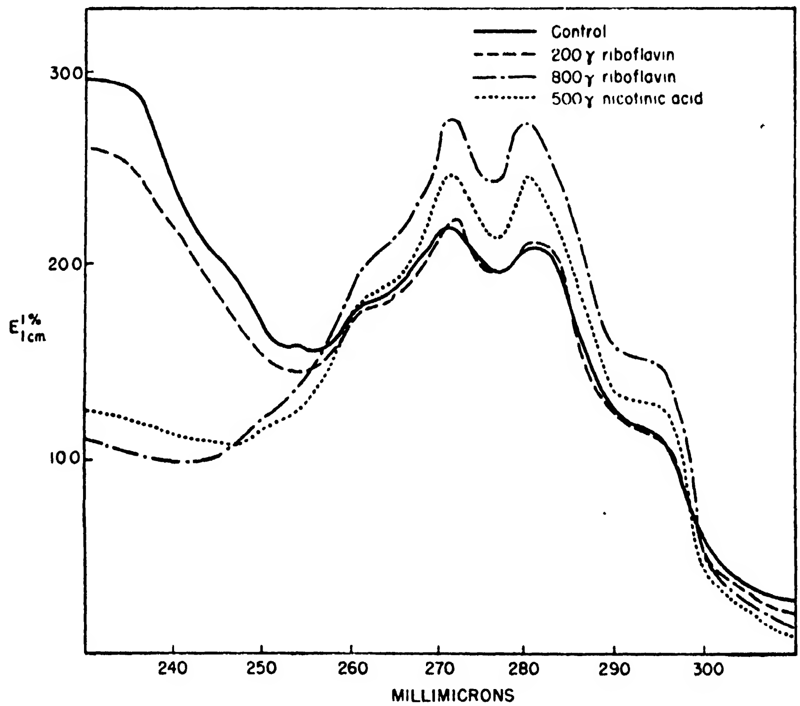

FIG. 1. UV Curves of FlB fats shown in Table II. ————Control; – – – – – 200 γ riboflavin; —·—·—· 800 γ riboflavin; ·········500 γ nicotinic acid.

From Fig. 1 it can be seen that there are marked changes in the UV curves. The fat obtained from FlB with the highest amount of added R and added NA showed the most significant deviations from those of the control, both in the range 230-265 mμ and 265-310 mμ. In addition, these 2 samples had the highest I.V.'s (Table II). A compari-

son of the changes in the lower range of the UV curve (230–265 mμ) with that obtained by adding S to FlB (2) reveals a very close similarity. However, in the other region, in contrast to that obtained with S, there are deviations in the curves indicating a change in the ergosterol content of the various fats analyzed; *e.g.*, the higher the absorption, the greater the sterol content of the fat.

These findings demonstrate that R and NA can function in the dehydrogenations involved in the formation of unsaturated fats in this mold. Thus, supporting evidence is presented for the conversion diagram discussed in an earlier communication from this laboratory (5).

B. Experiments with Flyco

It has been shown that S had no appreciable effect upon the composition of the fat produced by the pigmented Flyco (2). Since Flyco could be obtained both pigmented and unpigmented, it was of interest to study what effect riboflavin and nicotinic acid may have on fat formation in these molds.

Both the pigmented and unpigmented Flyco were grown in the dark on a Raulin-Thom medium containing 5% glucose. The analytical procedures were the same as those described for FlB. In addition, the pigment content of the mycelium was determined by the following method:

Pigment Determination. The mycelium was suspended in 100 ml. of 10% HCl for 24 hr. At the end of this time, the acid was decanted, the residue washed with distilled water until acid-free, and dried at 40°C. for 24 hr. A weighed sample was then extracted in a Soxhlet with $CHCl_3$ for 7 days. The extracts, properly diluted, were then read in an Evelyn photoelectric colorimeter, using a 520 mμ filter. Pure lycopersin (6) was used as the standard.

Effects of Riboflavin and Nicotinic Acid on Fat Formation in Pigmented Flyco

The results of these experiments are presented in Table III and in Fig. 2.

The addition of the two vitamins, like S, causes no change in the I.V.'s of the isolated fats from the control value; on the other hand, unlike S, there is an increase in mycelial weights, percentage of total lipides and fat coefficients (Table III). The fats were analyzed spectro-

TABLE III

Effects of Riboflavin and Nicotinic Acid on Fat Formation in Pigmented Flyco

Sample no.	Added substance	Mycelial wts.	Total lipide	Sterol in isolated fat	Iodine value	Pigment mg./g. mycelium	Riboflavin γ/g. mycelium	Fat coefficient
	γ/l.	g./l.	per cent	per cent				
1	Control	15.50	23.16	2.2	77	19.70	16	6.94
2	40 R.	16.79	24.69	1.7	75	17.85	14	8.29
3	200 R.	16.44	25.36	1.5	75	20.00	15	8.34
4	800 R.	17.39	25.69	1.1	75	18.00	17	8.94
5	500 N. A.	16.73	25.21	1.2	75	19.60	14	8.46

R. = Riboflavin; N. A. = Nicotinic Acid.

photometrically to determine what changes had occurred in the composition of the isolated fats which could not be detected by the I.V. Prior to the analysis, pigments present in the oil had to be removed since they, too, would absorb in the UV region. Clear, colorless oils were obtained by dissolving the fats in anhydrous ethyl ether and treating them with Florisil 3 times by simply shaking and decanting. Filtration of the ethereal solution and removal of the solvent *in vacuo* under nitrogen completed the process.

Substances other than the pigments were not removed, for the I.V.'s of a pigmented and depigmented sample were 77 and 78, while their percentages of sterol were 2.2 and 2.3, respectively (Sample 1, Table III).

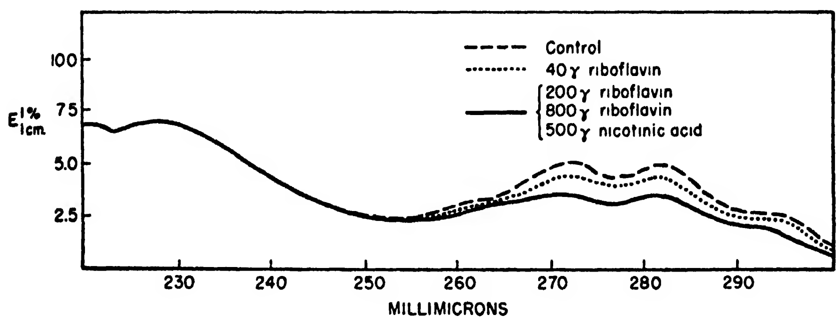

FIG. 2. UV Curves of pigmented Flyco fats shown in Table III. ——————— Control; .... 40 γ riboflavin; ———————200 γ riboflavin, 800 γ riboflavin, 500 γ nicotinic acid.

It was found that the absorption curves of the depigmented fats (Fig. II) were identical in the range 220–260 mμ, with only slight changes in the region 260–310 mμ. These changes are due to variations in the sterol content. This was confirmed by sterol analysis on the fats (Table III).

In the case of pigmented Flyco, as with FlB, the added vitamins cause no change in the R content of the mycelium from their respective controls. In view of subsequent findings, it must be borne in mind that the quantity of R in pigmented Flyco (15 γ R/g.) is half the amount found in both FlB and unpigmented Flyco (30 γ R/g.).

Both R and NA do not influence the quantity of pigment formed by this mold.

From consideration of the previous data, attempts were made to establish differences in the effects of the vitamins on pigmented and unpigmented Flyco, and to determine whether they would affect unpigmented Flyco in a manner analogous to their effect on FlB.

Effects of Riboflavin and Nicotinic Acid on Unpigmented Flyco

These results are recorded in Table IV and Fig. III. There seems to be no correlation between the amounts of R and NA added and their effects on the I.V., percentage of total lipide, and fat coefficient.

The oils of the unpigmented Flyco had a red tint. By dissolving a small portion of the various samples in $CHCl_3$, and determining $E^{1\%}_{1cm.}$ at 522 mμ with a Beckmann spectrophotometer, only 0.1% pigment was found in the most highly colored oil. This amount would not interfere with the spectral analyses of the fats, since several dilutions must be made prior to the spectrophotometric determinations.

As shown in Fig. 3, the spectral curves of all samples tested differ from each other. It can be seen that the oil with the highest I. V. (134) had the highest absorption in the 265–310 mμ range, while that with the lowest I.V. (103) had the lowest absorption in this region. Sterol analyses on the two samples showed that Sample 2 (I. V. 134) contained 6.4% sterol, whereas Sample 3 (I. V. 103) contained 3.4%. Therefore, the changes between 265–310 mμ are again due to the sterol contents of the samples. A possible explanation for the rise and fall of the I. V.'s and other data of Table IV could be that, although unpigmented Flyco does not produce appreciable amounts of lycopersin, sufficient quantities of it were formed to counteract the effects of the vitamins. Quantitative pigment determinations showed this to be the case (Table IV).

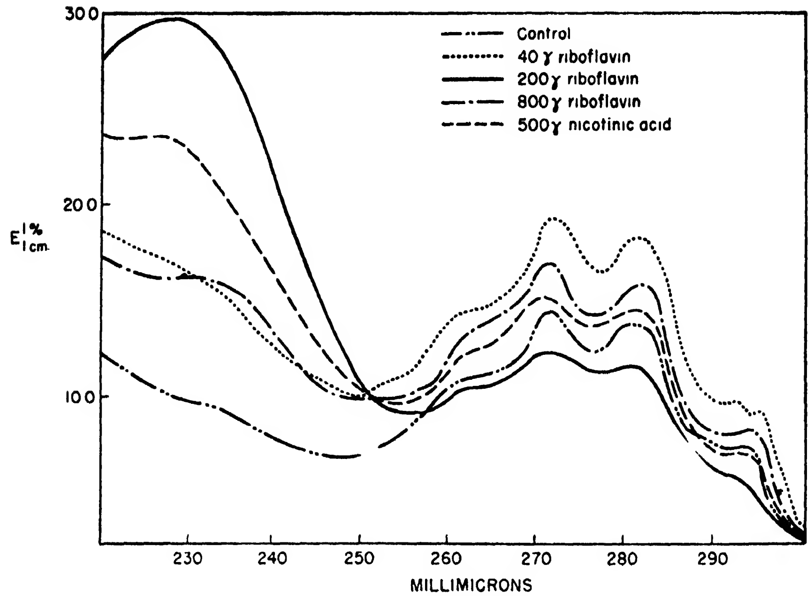

FIG. 3. UV Curves of unpigmented Flyco fats shown in Table IV. ·—·—·— Control; ············ 40 γ riboflavin; ————200 γ riboflavin; ·—·—·— 800 γ riboflavin; – – – – – –500 γ nicotinic acid.

The following facts explain the differences in the control values obtained with both FlB and pigmented and unpigmented Flyco (Tables II, III, IV, respectively):

(1) The metabolism of Flyco when grown unpigmented seems to approach the metabolism of FlB, while it is very unkile that of pig-

TABLE IV

Effects of Riboflavin and Nicotinic Acid on Fat Formation in Unpigmented Flyco

Sample no.	Added substance	Mycelial wts.	Total lipide	Iodine value	Pigment mg./g. mycelium	Riboflavin γ/g. mycelium	Fat coefficient
	γ/l.	g./l.	per cent				
1	Control	3.95	4.35	118	0.12	30	0.32
2	40 R.	3.84	4.47	134	0.26	30	0.34
3	200 R.	4.10	7.35	103	1.09	29	0.60
4	800 R.	4.34	5.43	123	0.45	32	0.47
5	500 N. A.	3.70	6.53	107	1.05	30	0.53

R. = Riboflavin; N. A. = Nicotinic acid.

mented Flyco, *e.g.*, FIB and unpigmented Flyco have low mycelial weights, percentage of total lipides, and fat coefficient, together with high I.V. and R content as compared to pigmented Flyco.

(2) The R content of unpigmented Flyco and FIB is twice the amount found in pigmented Flyco. It appears, therefore, that, at a high pigment level, there is low R production resulting in a fat with a low I.V. (77); and that, in the case where no appreciable amount of pigment is produced, the R production increases and so does the I.V. of the fat (118). This lends support to the concept that the vitamin is part of the dehydrogenating system involved in the production of unsaturated fats, and that the pigment and the vitamin may be antagonistic to each other, *i.e.*, one tending to raise the desaturation and the other to lower it.

(3) On comparing the pigmented and unpigmented Flyco, the evidence for previous work (8) is borne out vividly. It had been found that, when various pigment-producing *Fusaria* were grown under different conditions, maximum fat production coincided with maximum pigment production and maximum fat coefficient. In this experiment the conditions were ideal, for the same strain could be grown under identical conditions and yet be different in the most important respect which we had been trying to explain, *i.e.*, the interrelationship of fat and pigment production. Thus, when the pigment is produced, the percentage total lipide (25%), the pigment production (20 mg.), and the fat coefficient (7), are extremely high as compared to the non-pigment-producing Flyco, *i.e.*, percentage of total lipide = 4%, pigment production = 0.12 mg., and fat coefficient = 0.32.

On the basis of the above information, the wide deviations in Table IV can be explained as follows: (1) With 40 γ R added, the I.V. rises, as was the case with FIB. Very little increase in pigment production is noted. (2) With 200 γ R added, one would expect to find at least the same effects as observed with 40 γ R. But this sample has an even lower I.V. than the control. Attention must be directed to the fact that the amount of pigment produced in the mycelium is more than 4 times that of Sample 2. This is further demonstrated in the cases of Samples 4 and 5, where high pigment production is accompanied by a low I.V., and *vice versa*. Hence, it would appear that there is a very sensitive antagonism between the pigment and the added vitamins. (3) With Sample 3, maximum fat and pigment production and maxi-

mum fat coefficient occur in the course of adding R in increased amounts. Thus, it most resembles pigmented Flyco in its metabolism.

These analytical data are good evidence for the fact that lycopersin is antagonistic to the action of R and NA in the production of unsaturated fats. Such effects of R in the inhibitions of dehydrogenating systems by drugs were noticed by Sevag (17a,b), which lends further support to our findings.

These observations also afford an answer as to why the vitamins have no effect on the degree of desaturation of the oils obtained from pigmented Flyco. Apparently, to cause a rise in the I.V. of these fats, a considerable quantity of the vitamins would have to be added to overcome the effects of the large amounts of pigment present.

C. Linolenic Acid in Fusarium Fat

Previous analyses, using chemical procedures, indicated that no linolenic acid was detectable in the fat of FlB, and that the 2 main unsaturated fatty acids present were oleic and linoleic acids (9). More detailed studies followed, confirming the earlier findings, and, in the case of Flyco, showed also that linolenic acid was lacking in its isolated fat (2).

In the present studies an explanation was required as to what constituents other than sterol changed in the composition of the fats to account for the differences noted in the I.V.'s.

The first step was to establish the presence or absence of linolenic acid in those fat samples obtained by adding R and NA, and to compare them with the respective controls in which linolenic acid was not found. For this purpose, a spectrophotometric procedure, which is far more sensitive than chemical methods, was employed.

Brice and Swain (18) pointed out that the spectrophotometric method of Mitchell *et al.* (19) involved difficulties when only small proportions of conjugated components are present after isomerization. Both methods measure linolenic acid at 268 mμ. In the vegetable fats (*e.g.*, those of *Fusaria*) the presence of ergosterol, having one absorption maximum at 271 mμ would definitely interfere in these determinations. Both methods (18,19) are, therefore, inapplicable in such cases unless the sterol is removed before the final UV absorption readings are taken. The method now employed in this laboratory is an improvement of that suggested by Brice and Swain.

Detection of Linolenic Acid in the Presence of Ergosterol

The preparation of reagents and the isomerization procedure were identical to those described (18). The treated sample was then dissolved in 50 ml. of methanol and 100 ml. of water added. This solution was extracted with two 100 ml. portions of ethyl ether to remove sterol, acidified with H_2SO_4 and again extracted with three 50 ml. portions of ethyl ether. After washing the ether extract with water until acid-free, it was dried with anhydrous Na_2SO_4, and filtered through a sintered glass funnel. The residue obtained after removal of the solvent *in vacuo* under nitrogen was dissolved in 11 g. of the KOH-glycerin reagent, and transferred quantitatively to a 50 ml. volumetric flask with methanol. After adjusting the subsequent dilutions so that the observed densities lay between 0.2 and 0.8, the UV readings were taken between 260 and 280 mμ (the blank must be carefully diluted with methanol to the same extent as the sample).

In Fig. 4 is presented the evidence for the statement that ergosterol must be removed before making the final UV absorption analysis on fat

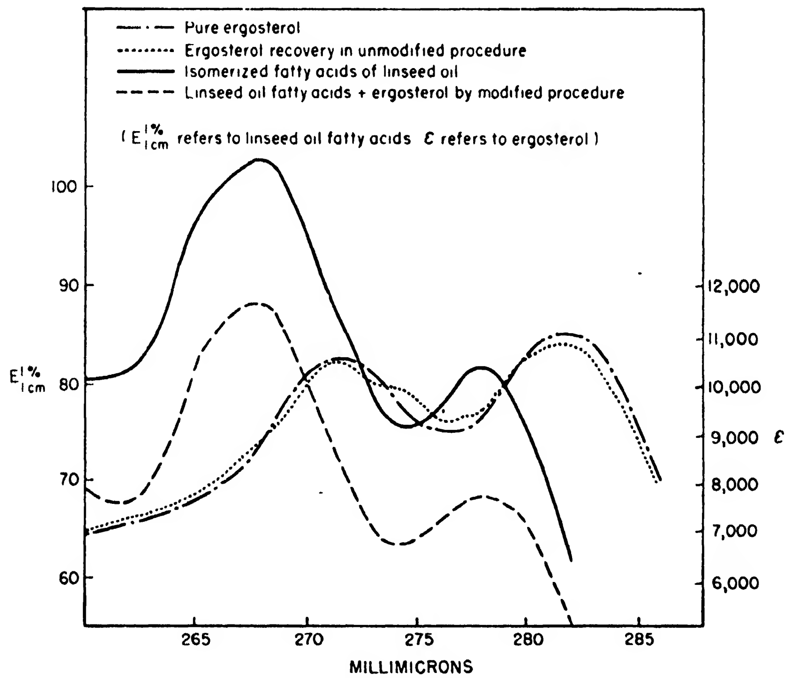

FIG. 4. UV Curves demonstrating interference of ergosterol in spectral detection of linolenic acid. —·—·—·— Pure ergosterol; ············ ergosterol recovery in unmodified procedure; ————————isomerized fatty acids of linseed oil; — — — — — linseed oil fatty acids + ergosterol by modified procedure. ($E^{1\%}_{1cm.}$ refers to linseed oil fatty acids; ϵ refers to ergosterol.)

samples that contain the sterol. It will be noticed that, if the absorption spectrum of pure ergosterol [obtained from *Fusaria* (9)] is taken before and after isomerization by the original method, almost 100% recovery of the sterol can be obtained. This means that the sterol remains unaffected in the procedure, and that its typical curve would be obtained despite such treatment. Therefore, ergosterol, if present in a fat containing small quantities of linolenic acid, would undoubtedly interfere with the spectrophotometric determination of the acid (especially since the sterol content may be as high as 10%).

To show the validity of the improved method, two samples of linseed oil fats, with and without added ergosterol, were taken through the altered isomerization procedure. Ergosterol was completely removed by this method, for in both cases a similar curve was obtained.

Linseed oil fatty acids instead of linolenic acid were used as reference substances in these determinations in order to simulate conditions qualitatively approaching those prevailing in *Fusarium* fats.

Linolenic acid was identified by the modified procedure in the controls of FlB and pigmented and unpigmented Flyco. This demonstrates the limitations of the original procedure.

The approximate linolenic acid content of the samples was determined by comparison of the $E_{1cm.}^{1\%}$ of the acid at its absorption peak at 268 mμ to that of the linseed oil fatty acids, assuming these to consist of 49% linolenic acid (20). The following observations were made:

(1) The FlB control fat contained approximately 10% of the trienoic acid. The other fat samples (Table II, 2–5) were combined, and the combined samples showed approximately 10% linolenic acid. (2) The control fat of pigmented Flyco showed approximately 1% of the acid, and the combined fat samples (Table III, 2–5) contained the same amount of linolenic acid. (3) Unpigmented Flyco control fat contained approximately 9% and the combined fat samples (Table IV, 2–5) approximately 4% linolenic acid.

These results indicate that:

(1) The increase in I.V. (77) in pigmented Flyco control fat to 118 in unpigmented Flyco control fat can be attributed to an increase in linolenic acid (*i.e.*, from 1% to 9%, respectively). Thus, in the presence of the pigment, less of this acid is synthesized in the mold. This relationship is extremely sensitive, as is shown in a further observation. R and NA had no effect on the linolenic acid formation in FlB and

pigmented Flyco, but in unpigmented Flyco, as the amount of pigment produced fluctuated, the quantity of the linolenic acid varied (*e.g.*, from 9% to 4%).

(2) The vitamins, therefore, cause no change in the linolenic acid content of the FIB or pigmented Flyco, and, in the case of unpigmented Flyco, are antagonized by the slight pigment production resulting in a lower linolenic acid production.

Comments

The evidence presented shows that R and NA, when added to FIB in different amounts, cause the formation of fats of varying degrees of unsaturation. Also, it was clearly demonstrated that both vitamins can participate in the dehydrogenations involved in the carbohydrate→fat conversion in this mold. The alteration in the composition of the fats of FIB in the presence of these two agents consists of an increase in the sterol content, and a change in the oleic: linoleic acid ratio. That the excess of desaturation should be considered as an alteration of this ratio can be deduced from the fact that no change in the linolenic acid level was found.

With pigmented Flyco practically no change occurs in the composition of its isolated fats when grown with added R and NA. This observation suggests that the presence of the pigment antagonizes the expected effects of the vitamins, as was found with FIB. This interpretation is further strengthened in the case of unpigmented Flyco, where, with added vitamins, the results obtained are, in some cases, similar to those of FIB, while, in other cases they resemble those of pigmented Flyco. This is due to the production of very small amounts of lycopersin by the unpigmented cultures. Unlike the findings in FIB, here the changes in fat composition are due primarliy to sterol content and linolenic acid.

The data presented corroborate the initial findings (5) regarding the postulated desaturation of saturated and unsaturated fatty acid.

Summary

1. Riboflavin has been found to be present in *Fusarium lini* Bolley.

2. Riboflavin and solanione affect the formation of fat in *Fusarium lini* Bolley with the same net result, but by different mechanisms.

3. Riboflavin and nicotinic acid are capable of taking part in the

mechanism of enzyme action operative in the formation of unsaturated fats in *Fusaria*.

4. Riboflavin and nicotinic acid, when added to *F. lini* Bolley and pigmented and unpigmented *F. lycopersici*, affect their fat formation in varying ways, *i.e.*, they increase the desaturation of fats produced by *F. lini* Bolley, exert no effect on the fat of pigmented *F. lycopersici*, and have a varying effect on the fat of unpigmented Flyco depending on the amount of pigment produced.

5. Lycopersin seems to antagonize the effect of R and NA on fat formation.

6. Linolenic acid is present in the fat of *F. lini* Bolley and pigmented and unpigmented *F. lycopersici*.

7. The observed changes in iodine absorption values in the fats of *F. lini* Bolley (grown with added R and NA) are due to an alteration of the amount of sterol and linoleic acid content; those in the fats of unpigmented *F. lycopersici* involve a change in sterol and linolenic acid content.

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Lack of Effect of Carbamyl-L-Glutamic Acid on the Growth of Certain Arginineless Mutants of *Neurospora*

Studies of arginineless strains of *Neurospora* have indicated that the terminal stages of the biosynthesis of arginine in this organism correspond, in outline form at least, to the sequence of reactions postulated by Krebs and Henseleit for an ornithine cycle in the mammalian liver (1,2). With the demonstration by Cohen and Grisolia (3) that carbamyl-L-glutamic acid acts as an intermediate in the synthesis of citrulline from ornithine in rat liver homogenates it became directly of interest to determine whether any of the available arginineless mutants of *Neurospora* are unable to make their arginine because of a failure to synthesize carbamyl-L-glutamic. In this regard, it was particularly pertinent to determine the effect of carbamyl-L-glutamic acid on strains whose growth requirement for arginine can also be satisfied by citrulline but not by ornithine. This is because mutants of this type presumably represent cases where gene mutation has given rise to an inability to convert ornithine to citrulline. Two such strains which differ genetically from one another, 30300 and 33442, have already been described in the literature (1), and a third strain, 30820, which is genetically diverse from either of the other two, was also at hand for test purposes.

Experiments in which carbamyl-L-glutamic acid was tested as a possible growth factor for arginineless mutants showed that this compound neither appreciably stimulated nor inhibited growth of the mutants under conditions usually employed in culturing *Neurospora* in liquid medium. These conditions are described in detail elsewhere (1). The results of a typical experiment are shown in Table I.

The experiment summarized in Table I was repeated with the media being buffered at pH 4.5 and 6.5, and was also done at 31°C. In each case, the results were essentially those already shown; that is, carbamyl-L-glutamic acid did not promote growth of the strains under investigation. In addition, strain 36703, which has a specific amino acid requirement for arginine, and strain 29997, which grows on ornithine

TABLE I

Growth of Mutant Strains

Values represent dry weight in mg. of mold grown on minimal medium and on minimal medium supplemented with various amino acids. Experimental cultures grown in 125 ml. Erlenmeyer flasks. Total medium per flask: 20 ml.; sucrose concentration: 2%. Arginine, citrulline, or ornithine when present: $M/6400$. Carbamyl-L-glutamic acid when present: 5 mg. per flask. Temperature: 25°C. Mycelia of strains 30300 and 33442 harvested at the end of 3 days; mycelia of strain 30820 harvested after 5 days.

Supplement to minimal medium	Strain 33442	Strain 30300	Strain 30820
Arginine	24.6	21.5	12.0
Citrulline	25.8	18.4	16.3
Ornithine	4.2	0.0	0.0
Carbamyl-L-glutamic	5.0	0.0	0.0
Arginine plus carbamyl-L-glutamic	24.5	22.1	13.8
Ornithine plus carbamyl-L-glutamic	6.0	0.0	0.0
No supplement	6.3	0.0	0.0

besides growing on citrulline or arginine, were grown in the presence of carbamyl-L-glutamic acid, with no effect of this compound being shown.

These results are not to be taken as signifying that carbamyl-L-glutamic acid plays no role in the synthesis of arginine in *Neurospora*, but may mean only that no mutant strains have yet been isolated which represent blocks in the synthesis of the former compound.

Acknowledgments

The mutant strains of Neurospora dealt with in this paper were originally obtained for study by the uthor from Dr. G. W. Beadle and Dr. E. L. Tatum at Stanford University in 1943. The carbamyl-L-glutamic acid used in these experiments was kindly supplied by Dr. Philip P. Cohen of the University of Wisconsin. Thanks also are due to Mr. Shigemi Honma, a student in the Cornell laboratories, who performed patient and arduous work in reisolating strain 33442 after it had been lost through contamination of stock cultures.

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n-Diamylacetic acid and Nitrate Assimilation

Data have been presented showing that n-diamylacetic acid has a very pronounced influence on the growth behavior of roots (1) that is not quite consistent with its action in the pea test (2). In the concentration 10^{-5} M it causes an inhibition of the elongation of the root epidermis but not of the inner parts of the root. To a certain extent, it has an action antagonistic to that of a synthetic auxin, naphthylacetic acid. It was emphasized, however, that it is, nevertheless, premature to assume that n-diamylacetic acid in roots only acts as an antiauxin.

To investigate whether this acid directly affects the growth mechanism or some part of the metabolism, its action was studied on the absorption and assimilation of nitrate together with the sugar consumption. The experiments were performed on excised, aseptic roots of wheat and run for 48 hr. Further particulars, together with a full account of the results, will appear in *Physiologia Plantarum.* Only some preliminary data on the nitrate consumption will be discussed here.

TABLE I

Absorption and Consumption of Nitrate and Glucose as Influenced by n-Diamylacetic Acid

(The figures give μmol on 300 root tips)

D. A. A. mol	Nitrate		Glucose	
	Absorbed	Assimilated	Absorbed	Consumed
0	56	34	151	138
10^{-7}	37	14	116	111
10^{-6}	27	2	110	106
10^{-5}	31	1	130	122

Table I shows the results of one typical experiment with increasing additions of n-diamylacetic acid to a complete nutrient medium containing Fe and Mn, each 1/50,000 M. It was found that the acid constantly decreases the assimilation of nitrate, even in a concentration of 10^{-6} M or lower. In such a concentration, the acid has no visible influence at all on the cell elongation, and also the glucose consumption is but little affected. A reference should be made to the observation of Nance (3) that 2,4-D inhibits the absorption of nitrate, but since he had no assimilation in his material, a full comparison is impossible.

According to earlier investigations on wheat roots (4), assimilation of nitrate is promoted by additions of Mn, or, to a lesser extent, by Fe. The same holds true of roots poisoned by *n*-diamylacetic acid (Table II). An increasing supply of Mn or Fe restores the nitrate assimilation, as verified in a number of experiments. The concentrations in Table II are high, about 5 p.p.m., but the short duration of the experiments ensures that they are well below the toxic level.

TABLE II

Influence of Fe, Mn, and Mo on the n-Diamylacetic Acid Inhibition

(D. A. A. 10^{-6} mol. Figures as in Table I)

Addition			Nitrate	
Fe	Mn	Mo	Absorbed	Assimilated
0	0	0	33	5
10^{-4}	0	0	45	25
0	10^{-4}	0	42	15
10^{-4}	10^{-4}	0	42	21
0	0	0	26	5
0	0	$3 \cdot 10^{-7}$	26	7
0	0	$3 \cdot 10^{-6}$	23	6
0	0	$3 \cdot 10^{-5}$	19	0

Mulder (5), especially, has emphasized the importance of Mo for nitrate assimilation. As far as the results go at present with the poisoned roots, Mo alone does not restore the nitrate assimilation (example Table II). Higher concentrations of Mo are toxic.

From the fact that the inhibition of the nitrate assimilation appears at a much lower concentration of *n*-diamylacetic acid than the growth disturbances, the conclusion is to be drawn that the former represents a primary action of the acid. The neutralizing effect of Fe and Mn indicates an effect of the acid on some point within a redox system, either the acid directly encroaches upon the nitrate assimilation mechanism or upon some system connected with this process.

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Functions of ATP and Other Phosphoric Acid Derivatives

Functions of ATP reported to date include its participation in phosphorylation reactions, its role as an energy donor in enzymatic acetylations, a possible part in the formation of TPN and a role in the conversion of myosin to actomyosin. The phenomena are known, although their mechanisms are not. The importance of inorganic materials for enzymatic processes becomes ever more evident (1). Among such processes are those in which ATP plays a part.

In experiments which will be described more fully at a later date, we have observed a remarkable capability of ATP to dissolve insoluble metal salts and to prevent their normal precipitation. In the presence of a $M/5$ solution of sodium adenosine triphosphate, at a pH equal to or higher than 7, the carbonates and phosphates of Mg, Ca, Zn, Mn, Cu, Co, Ni, Fe^{++}, Fe^{+++} will not be precipitated. The same applies to the phosphates of Al, Cr and VO^{++}, the precipitation of $BaSO_4$, $CaSO_3$, Ca borate and, for a shorter time, also Ca silicate and Ca oxalate. CaF_2 and Ca stearate form clear solutions on standing. ATP prevents the precipitation of the sulfides of Mn, Ni, Co, Fe^{++}, Fe^{+++}, Cu and UO_2^{++} equally well, and ZnS "solutions" remain clear for a short time. There is no effect in the case of CdS. The strong complexes, Turnbull's blue and Hatchett's brown, are decolorized by ATP on warming and ferric thiocyanate is decolorized in the cold.

On the whole, the behavior of ATP is analogous to that recently described for nucleic acids (2). It is worthy of note that the neutral sodium salt of ATP also dissolves the compounds formed between nucleic acids and bi- or polyvalent metals as well as proteins, *e.g.*, ribo- and desoxyribonucleates of Ca, Fe^{++}, Fe^{+++}, ovalbumin, bovine plasma albumin, lysozyme, pepsin, *etc.* These abnormal solubilizing effects are so well defined that they may be expected to have certain functions in the course of biochemical processes. If some form of complex formation

is postulated as ultimate cause, it must be considered that it "catalyzes" reactions which otherwise would not take place (Meerwein).

The inorganic mother substance of ATP is triphosphoric acid, $H_5P_3O_{10}$. Its alkali salts have been shown (3) to have unusual solubilizing effects of a similar character. As yet, no triphosphate has been found in nature. The occurrence of two other condensed phosphoric acids, meta- and pyrophosphoric acids, has, however, been described. Since Liebermann in 1888 discovered metaphosphate in yeast it has repeatedly been observed in microorganisms, associated with nucleic acids (Liebermann, Ascoli and others). Pyrophosphate is present in cells, possibly as a cleavage product of polyphosphates. Hardly anything is known regarding the function of inorganic pyrophosphate. In the case of metaphosphate, some influence on mitosis (Lindegren) and a role during protein synthesis (Juni, Kamen, Reiner and Spiegelman) have been considered. Soluble double salts of meta- and pyrophosphates, respectively, have been reported in the literature. They are formed by dissolving bi- or polyvalent metal salts of meta- or pyrophosphoric acid in the alkali salts of the same acid. These solutions are assumed to contain complex metallophosphato anions. The presence of such complexes forms the basis of the technical application of these materials. Apparently, however, no systematic investigations have been made on the solubility of insoluble salts of acids other than meta- or pyrophosphoric in meta- and pyrophosphate. Such experiments have now been made and have demonstrated that insoluble carbonates, silicates, orthophosphates, arsenates, molybdates, borates, sulfates, sulfites, oxalates, phytates, pectates and nucleates of the previously mentioned metals, as well as those of Be, La, Cd, Pb, Sn, Th, Hg^{++}, UO_2^{++} and others, "dissolve" in meta- and pyrophsophate. Even solid $MgNH_4PO_4$ and ammonium phosphomolybdate, the latter in weakly acid solution, "dissolve" in both meta- and pyrophosphate. $BaSO_4$, CaF_2, Ag_2CO_3, Ag_3PO_4, Hg_3PO_4, *etc.*, together with sulfides and mercaptides of the biometals are either brought into solution or their precipitation is prevented. Protein, protamine and streptomycin nucleates are soluble in meta- and pyrophosphate; ferric thiocyanate, Prussian blue and Turnbull's blue yield colorless clear solutions, neutral Ca, Ba and Pb fructose-1,6-diphosphates dissolve and can no longer be reprecipitated by heat. Numerous insoluble pyrophosphates are brought into solution by $NaPO_3$ and, *vice versa*, insoluble metaphosphates dissolve in $K_4P_2O_7$. Salts of metaphosphoric acid with primary, secondary and tertiary

amines, amino alcohols and diamines sometimes exceed $NaPO_3$ in their solubilizing power. In all cases $NaPO_3$ refers to the soluble crystalline sodium hexa-metaphosphate. X-ray diffraction patterns have been reported for 6 different metaphosphates, and others seem to be known (4). There are indications of the occurrence of two or more distinct metaphosphates in cells (Macfarlane, Mann and others). ATP, and probably also the 3 purely inorganic condensed phosphoric acids mentioned above, are substances in which energy is being stored. This fact can hardly be connected with the solubilizing phenomena described, since we observed exactly analogous effects in the case of differently constituted phosphoric acid derivatives, such as alkali α- and β-glycerophosphate and 3-phosphoglycerate, alkali phytates, and Na and Mg fructose-1,6-diphosphates. A detailed account of these experiments is in preparation.

All these solubilizing agents are substances of the utmost biochemical importance, some of them are omnicellular, all of them are subject to degradation and transformation by widely distributed and specific enzymes. The appearance, disappearance and reappearance of these materials, characterized by their extraordinary solubilizing capabilities opens a series of new vistas on their functions within the cell, for the mobilization, transportation and sedimentation of insoluble substances in tissues and in the soil.

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Fate of S^{35}-Na Estrone Sulfate in Pregnant and Non-Pregnant Rats [1]

The metabolism of S^{35}-Na estrone sulfate[1] was studied in pregnant and non-pregnant rats. In addition, it was hoped that this study would

[1] We wish to thank Drs. Edward C. Reifenstein, Gordon A. Grant and J. Murray Scott of Ayerst, McKenna and Harrison, Ltd., N. Y., for their interest in the investigation and for supplying us with generous quantities of S^{35}-Na estrone sulfate.

give information relative to the fetal metabolism of a conjugated estrogen.

Adult female rats of the Sprague-Dawley strain were used. These animals were fed *ad libitum* and were kept in matabolism cages throughout the experiments. The S^{35}-Na estrone sulfate was given by subcutanoeus injection. The dosage used and length of experiments are given in the table. Urine and feces were collected up to 24 hr. after the last administration of labeled estrone sulfate. The pregnant rats were sacrificed on the 20th day, and the following tissues and organs were analyzed for their S^{35} content: blood, liver, spleen, pancreas, adrenals, kidneys, ovaries, uterus, small intestine, large intestine, cecum, placentae and fetuses. In the experiments on the non-pregnant rats, only the blood, urine, and feces were analyzed. The S^{35} content of the various fractions was determined by the method of Tarver and Schmidt (1). All radioactive measurements were carried out by conventional counting methods.

TABLE I

Distribution of S^{35} in the Urine and Feces of Pregnant and Non-Pregnant Rats After Subcutaneous Injection of S^{35}-Na Estrone Sulfate

The S^{35}-Na estrone sulfate was administered in aqueous solution, pH 7.4

Experiment	Number of animals	Injection schedule	Total injected dose		Total S^{35} present in				Per cent recovery
					Urine		Feces		
			mg.	Counts S^{35}/min.	Counts S^{35}/min.	Per cent of injected dose	Counts S^{35}/min.	Per cent of injected dose	
Pregnant									
I	5	One injection every 24 hr. on 17, 18, 19 day	3.2	530,000	175,000	32	220,000	42	74
II	5	One injection every 24 hr. on 17, 18, 19 day	5.4	870,000	200,000	20	460,000	53	73
III	2	One injection every 24 hr. on 17, 18, 19 day	2.0	322,000	74,100	23	177,100	55	78
IV	3	One injection every 24 hr. on 17, 18, 19 day	3.0	483,000	130,000	27	207,700	43	70
Non-pregnant									
I	6	One injection every 24 hr. for 2 days	6.5	1,030,500	288,500	28	443,400	43	71
II	3	Single injection	1.6	250,000	58,300	24	110,000	44	68
III	3	Single injection	1.6	250,000	67,600	27	102,000	40	67

In the pregnant rats, no S^{35} was found in any of the tissues or organs examined, and, in particular, no evidence of any S^{35} was found in the placentae or fetuses. Thus, it appears that no S^{35}-Na estrone sulfate or any S^{35} that might be liberated from this compound crossed the placental barrier.

Over 70% of the total amount of S^{35} injected into pregnant rats and non-pregnant rats was found in the urine and feces; approximately two-thirds was present in the feces and approximaetly one-third in the urine. Preliminary evidence has been obtained on the rate of excretion of S^{35} from animals injected subcutaneously with S^{35}-Na estrone sulfate. Approximately 90% of the total amount of the S^{35} excreted *via* the kidney appeared in the urine within 12 hr., and 100 % in 24 hr. In the same animals, approximately 80% of the total S^{35} excreted *via* the intestinal tract was found in the feces in 24 hr. and 100% in 48 hr. The total recovery of S^{35} from the urine and feces after 48 hr. was 80–85% of the injected dose.

The data indicate that the rat rapidly excretes S^{35}-Na estrone sulfate. Inasmuch as the excreted S^{35} was mainly present in the inorganic form (none detectable as estrone sulfate), it was apparent that the body must contain an enzyme capable of hydrolyzing this conjugated estrogen. The action of various tissue homogenates on S^{35}-Na estrone sulfate gave additional evidence in support of this enzyme system. Using the isotope dilution technique, it was observed that rat liver homogenate contains a sulfatase capable of hydrolyzing this conjugated estrogen. Further details on these experiments will be presented in a subsequent paper.

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The Isolation of a Crystalline Actinomycin-like Antibiotic

The production of actinomycin by different actinomycetes is being more and more frequently encountered (1–4). In the course of our studies, we found several actinomycetes producing appreciable amounts of a crystalline antibiotic which we believed to be identical with actinomycin. The availability of over 100 g. of the crystalline material made possible the preparation of highly purified preparations, which,

surprisingly, gave certain analytical results not in agreement with those published for actinomycin (1). This fact and the simplicity of our isolation procedure prompt this letter.

A few widely different media have been found to give good yields of the antibiotic, but the best of those tested contains 2% soybean flour, 2% brown sugar, 0.5% cornsteep liquor, and 0.1% K_2HPO_4. In a series of ten 100 gal. tank batches charged with this medium, aerated submerged growth of our organism X-45 for 8–10 days at 25°C., with an airflow rate of 10 cu. ft. air/min., resulted in a broth potency equivalent to 0.125–0.25 mg. antibiotic/ml. *Sarcina lutea* was used as a test organism in seeded agar cup-plates for assaying the potency.

The antibiotic was isolated by stirring for 10 min. the filtered broth at pH 8.5 with half its volume of butanol. The separated butanol layer was dried over anhydrous Na_2SO_4, evaporated *in vacuo*, and the residual tar taken up in a minimum amount of warm ethanol. The filtered ethanol solution, upon concentration and cooling, deposited the antibiotic as a crystalline powder. The yields varied with the potency of the culture fluids and were between 40 and 80% of the total activity present in the broth. For further purification, the crystals were dissolved in cold chloroform, filtered from insoluble, inactive material, and the chloroform removed under reduced pressure. The residue, on recrystallization, gave red plates from ethanol, or orange needles from butanol; m.p. 250–252°C. (corr.) with decomposition.

Prep. I: C, 57.97; H, 6.47; N, 13.06;
$[\alpha]_D^{31} = -332°$ (c = 0.25, ethanol).
Prep. II: C, 57.75; H, 6.57; N, 12.86;
$[\alpha]_D^{31} = -340°$ (c = 0.25, ethanol).
Mol. Weight: 576 and 581 (Rast in camphor).
Spectrum: Maxima at 447 mμ ($E_{1\,cm.}^{1\%} = 200$)
and 240 mμ ($E_{1\,cm.}^{1\%} = 280$) in ethanol.

The isolated crystalline antibiotic was compared directly with an authentic sample of actinomycin, kindly supplied by Dr. M. Tishler of Merck & Co., against over 20 bacteria and fungi. Both preparations were found to be biologically identical within the limits of error of the cup plate assay employed. The toxicity in mice of our preparation was found to be of the same order as that reported in the literature (5). Other properties, such as color, melting point, optical rotation and absorption spectrum, were also in good agreement with the published

data. The results of the elementary analysis (carbon value) and the molecular weight of our preparations, however, differ from those reported by Waksman and Tishler for actinomycin (1). These authors found an average carbon value of 59.01% and a molecular weight of 768–1000.

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Effect of Insulin and Adenosinetriphosphatase on a Reaction Coupling Oxidation with Phosphorylation [1]

This is to report the *in vitro* effect of insulin on the production of high energy phosphate bonds during an aerobic oxidation.

The insoluble residue of a rat liver homogenate, which constitutes the "cyclophorase system" (1), has been demonstrated to be capable of catalyzing the oxidation of α-ketoglutaric acid and, concomitantly, to phosphorylate adenylic acid with inorganic phosphate. In the presence of yeast hexokinase, glucose, and NaF, inorganic phosphate is esterified to glucose phosphate through the pathway of high energy phosphate systems (ATP[2]). If the concentrations of yeast and liver enzymes are so adjusted that the rate of transphosphorylation between glucose and

[1] This investigation was supported in part by research grants from the U. S. Public Health Service and from Eli Lilly and Co.

[2] Adenosinetriphosphate.

ATP by the hexokinase system is always greater than the rate of ATP formation by way of the oxidative phosphorylation reaction catalyzed by the liver system, then any increase in the quantity of inorganic phosphate esterified must reflect a stimulation in the formation of high energy phosphate bonds by the " cyclophorase system." This condition was satisfied by the use of (a) less than optimal concentrations of ATP, (b) a liver enzyme of reduced activity (aged preparation), and (c) an excess of yeast hexokinase. The addition of insulin to this system resulted in a significantly increased rate of esterification (Table I) with approximately one out of every three enzyme preparations. In a series of 166 experiments performed with 77 different liver enzyme preparations and 20 hexokinase preparations, the addition of 0.02–0.2 units of insulin to the test system produced a statistically significant increase ($p = \ll 0.001$) in the quantity of phosphate esterified, an increase which averaged 1.4 micromoles phosphate (S.E. = 0.23 micromoles) over that produced in the absence of insulin. The lack of an appreciable stimulation of oxygen consumption is noteworthy.

TABLE I

The Effect of Insulin and Adenosinetriphosphatase on the Esterification of Inorganic Phosphate

Enzyme preparation	Incubation	Insulin	Mg ATPase	O_2 consumption	Inorganic phosphate esterified	Δ P^a
	(min.)	*(units)*	*(ml.)*	*(micromoles)*	*(micromoles)*	*(micromoles)*
14	15	0	—	5.7	3.5	—
		0.1	—	6.7	10.4	+6.9
	30	0	—	10.1	21.1	—
		0.1	—	11.0	24.1	+3.0
	45	0	—	13.8	21.4	—
		0.1	—	14.7	25.7	+ 4.3
56	30	0	—	11.3	15.6	—
		0.02	—	11.5	19.5	+3.9
		0.06	—	11.1	18.2	+2.6
		0.1	—	11.7	21.8	+6.2
1	30	—	0	12.6	34.0	
		—	0.04	10.0	32.0	−2.0
		—	0.06	9.4	25.0	−9.0
		—	0.1	6.5	4.5	−29.5
		—	0.2	5.2	2.0	−32.0

TABLE I—(*Continued*)

Enzyme preparation	Incubation	Insulin	Mg ATPase	O_2 consumption	Inorganic phosphate esterified	Δ P[a]
	(*min.*)	(*units*)	(*ml.*)	(*micromoles*)	(*micromoles*)	(*micromoles*)
2	30	0	0	8.5	5.6	
		0.06	0	9.1	8.1	+2.5
		0	0.02	5.4	0	
		0.1	0.02	6.7	2.5	+2.5
		0.2	0.02	7.0	3.8	+3.8
		0.4	0.02	7.9	5.3	+5.3
		0.6	0.02	8.2	5.5	+5.5
3	30	0	0	11.2	33.3	
		0	0.07	6.3	6.8	
		0.5	0.07	6.6	6.6	−0.2
		1.0	0.07	7.1	5.0	−1.8
		2.0	0.07	6.7	7.5	+0.7
		5.0	0.07	7.2	9.7	+2.9
4	30	0	0	13.5	31.4	
		0	0.04	11.7	19.3	
		0.5	0.04	11.0	18.2	−1.1
		2.0	0.04	12.9	23.7	+4.4
		2.0[b]	0.04	11.0	16.4	−2.9

Reaction mixtures incubated in Warburg vessels in a total final volume of 3.0ml. The final concentrations of reagents were: α-ketoglutaric acid, 0.02 *M*; phosphate, 0.01 *M*; $MgCl_2$, 0.007 *M*; NaF, 0.02 *M*; glucose, 0.01 *M*; ATP, 0.000433 *M*; histidine, 0.0025 *M*. 0.8 ml. of the liver homogenate in 0.2 *M* KCl, 0.02 *M* $NaHCO_3$ (4) together with 0.2 ml. of yeast hexokinase preparation (2) were placed in one side arm, insulin and ATPase when present, in the other. The pH was 7.2; temp. 28°C; gas phase, air. Inorganic phosphate was determined by the method of Fiske and SubbaRow (5).

[a] Increase or decrease in the amount of phosphate esterified in the presence of insulin and/or Mg ATPase.

[b] Inactivated by heating for 3 hr. at 37°C. in 0.05 *M* NaOH.

Experiments performed with the omission of yeast hexokinase or α-ketoglutaric acid or oxygen from the test system demonstrated little or no phosphorylation and no significant insulin effect. Since insulin is known to have no effect on yeast hexokinase (2), it may be concluded that the action of insulin in the described enzyme complex increased the formation of high energy phosphate bonds by an aerobic oxidation.

This effect of insulin was further investigated in an effort to increase the reproducibility and clarify the nature of the reaction observed.

The addition of a cyanide-free magnesium-activated adenosinetriphosphatase (Mg ATPase) (3) to the test system resulted in an inhibition of both the oxygen uptake and the quantity of inorganic phosphate esterified (Table I, Enz. Prep. 1). The addition of insulin to a Mg ATPase-inhibited system released the inhibition and resulted in a concomitant increase in oxygen uptake and phosphate esterified (Enz. Prep. 2). The effect of a given concentration of insulin on the ATPase inhibition varied with the activity of the ATPase in the liver homogenate. With lower insulin concentrations the inhibition with Mg ATPase was actually enhanced. As the concentration of insulin was increased, the inhibition was gradually released (Enz. Prep. 3). This may possibly be explained by the increased inhibition observed with insulin inactivated by a procedure that destroyed the hypoglycemic factor but not the hyperglycemic factor of commerical insulin[4] (Enz. Prep. 4).

When the concentrations of the ATPase and "cyclophorase" enzymes were so adjusted as to yield approximately a 50% inhibition in phosphorylation, the addition of insulin in sufficient amounts resulted almost invariably in the reversal of the inhibition produced by Mg ATPase.

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Synthesis of the Short-Chain Fatty Acids of Milk Fat from Acetate

The utilization of acetate *in vitro* by mammary gland slices from ruminants (cow, goat) (1) and non-ruminants (rat, rabbit) (2) with R. Q. > 1 has been interpreted as indicating milk fat synthesis from

acetate. The origin of the short-chain acids (C_4–C_{14}) of milk fat has long been disputed. The probability, however, that they originate from acetate was pointed out (1).

Experiments in pregnant rabbits provided further evidence for the *in vivo* utilization of acetate by the mamma for fatty acid synthesis (3). Most striking was the high rate of incorporation of C^{14} into glyceride fatty acids extracted from the mammae of pregnant rabbits injected with $CH_3C^{14}OONa$.

We have fractionated the glyceride fatty acids, isolated from the mammae of 5 of these rabbits, into steam-volatile (divided further into water-soluble and insoluble fractions) and non-volatile fatty acids.

TABLE I

C^{14} Content of Glyceride Fatty Acids from the Mammae and Livers of Rabbits 28 Days Pregnant

Fatty acid fraction	Rabbit no. 10		Rabbit no. 13		Rabbit no. 15	
	C^{14}/mg. fatty acid $\mu c. \times 10^{-4}$	C^{14}/mg. carbon $\mu c. \times 10^{-4}$	C^{14}/mg. fatty acid $\mu c. \times 10^{-4}$	C^{14}/mg. carbon $\mu c. \times 10^{-4}$	C^{14}/mg. fatty acid $\mu c. \times 10^{-4}$	C^{14}/mg. carbon $\mu c. \times 10^{-4}$
Mammary gland:						
Volatile, water-soluble	1.27	1.98	0.46	0.73	12.30	18.45
Volatile, insoluble	1.03	1.48	0.495	0.72	9.53	13.60
Non-volatile	0.09	0.12	0.05	0.07	0.75	0.99
Liver, unfractionated fatty acids	0.113	0.146	0.139	0.183	0.44	0.58

The C^{14}-content of the Na-salts was then determined. The results for 3 of the 5 experiments are shown in the table. In all 5 experiments the volatile acids contained 7–18 times more isotope than the non-volatile acids, and in 3 cases the water-soluble fraction (mainly butyric and caproic acids) had the highest C^{14}-content. The C^{14}-content of the liver glyceride fatty acids precludes the possibility that the highly active fatty acids in the mammae arise from liver fatty acids.

These results show, firstly, that the volatile acids in the mamma are synthesized there and are not derived by degradation of higher fatty acids and, secondly, that at least the carboxyl–carbon of acetate is specifically utilized in this synthesis. It seems highly probable that the short-chain fatty acids originate from condensation of C_2-units.

Since the results of these *in vivo* experiments could be predicted from the *in vitro* utilization of acetate by mammary gland slices it seems to us that the mammary gland is a particularly suitable organ for the study of the biochemistry of fatty acid synthesis.

It is not known in the present experiments what proportion of the fat was intracellular and what was present in stored secretion. Further studies of the mode of synthesis of fatty acids secreted in milk itself are in progress.

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Effect of Vitamin B_{12} and APF Supplement on Methionine Needs of the Pig

Cunha *et al.* (1) showed that an APF supplement (Lederle Laboratories) supplied an unknown factor(s) when added to a corn-peanut meal ration, whereas a vitamin B_{12} charcoal concentrate was of no benefit when added to the same ration. Burnside *et al.* (2) showed that the same APF supplement increased the feeding value of peanut meal and soybean oil meal so that these plant protein supplements were similar in feeding value to the fish meal used. The control ration used in this trial consisted of ground yellow corn 57, peanut meal 41.5, bone meal 0.5, limestone 0.5, salt 0.5%, plus vitamins A and D at levels of 5000 I.U. and 700 I.U., respectively, per pig daily. The other vitamins were added at the following levels per 100 lb. of feed: thiamine 1 g., riboflavin 230 mg., niacin 2.33 g., pantothenic acid 1 g., pyridoxine 375 mg., choline 19.4 g., and folic acid 22.7 mg. The control ration contained all the known vitamins which the pig has been shown to need (3). Four pigs were fed in each lot on concrete floors. The trial lasted for 6 weeks.

The APF supplement was of considerable benefit when added to the basal ration, which is in agreement with previous work by Cunha *et al.* (1) and Burnside *et al.* (2).

The vitamin B_{12} concentrate (Fuller's earth) was of little, or no help when added to the basal ration. This is in agreement with previous work by Cunha *et al.* (1) when a B_{12} concentrate (charcoal) was of no apparent benefit when added to the same basal corn-peanut meal ration.

Methionine was beneficial when added to the basal ration (Lot 2). However, when methionine was fed in addition to the APF supplement (Lot 3) the rate of gain was no different than when APF supplement was fed alone (Lot 4). Methionine was also of no benefit when added to the basal ration + vitamin B_{12}. It is difficult to explain why the rate of gain was lower when methionine plus B_{12} was added to the basal ration as compared to adding methionine alone (Lot 2). These data indicate

TABLE I

Lot no.	Av. starting weight	Ration fed	Av. daily gain
	lb.		*lb.*
1	21.0	Basal	0.52
2	20.9	Basal+0.3% methionine[a]	0.84
3	20.5	Basal+0.3% methionine+2.2% APF supplement[b]	1.11
4	21.0	Basal+2.2% APF supplement	1.14
5	20.6	Basal+0.1% B_{12} concentrate[c]	0.61
6	20.4	Basal+0.1% B_{12} concentrate+0.3% methionine	0.63

[a] DL-Methionine supplied through the courtesy of Dr. H. J. Prebluda, U. S. Industrial Chemicals, Inc., New York, N. Y.

[b] Animal Protein Factor supplement (fermentation product-N203B) obtained from Dr. T. H. Jukes, Lederle Laboratories, Pearl River, New York.

[c] Vitamin B_{12} concentrate (Fuller's earth) from Dr. D. F. Green, Merck & Co., Rahway, New Jersey (contained 12.5 mg. of B_{12} activity/lb.).

that the addition of the APF supplement to the basal ration relieved the need for supplementing the ration with methionine. This is in line with a recent report by Gillis and Norris (4), who showed that the inclusion of a source of APF in their basal diet relieved the need for supplementary methylating compounds (choline and betaine) for the chick.

The vitamin B_{12} concentrate and the APF supplement were similar from the standpoint that methionine did not help in growth when fed in addition to either of them. However, these concentrates differed, as shown by the fact that growth rate was appreciably increased only by the APF supplement. This may mean that vitamin B_{12} is a constituent

of the APF supplement and that the APF supplement contains another factor(s) of benefit for the pig.

These data indicate the importance of the Animal Protein Factor supplement in supplying an unknown factor or factors and its relationship to methionine and possibly protein needs of the young growing pig.

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The Effect of Adrenocorticotropic and Growth Hormones on the Glucose Uptake and Glycogen Synthesis by the Isolated Diaphragm with and without Insulin

In a previous communication (1) we reported that pretreatment with adrenocorticotropic hormone (ACTH) inhibits glycogenesis in isolated diaphragms of normal rats in the presence of insulin. Similar experiments using growth hormone have recently been carried out and it was found that the insulin effect in promoting glycogen storage is also impaired in the diaphragms of normal rats treated with growth hormone (Table I). It is of interest to note that the glucose uptake in both cases is not modified by the injection of either ACTH or growth hormone.

Experiments with isolated diaphragms of hypophysectomized rats gave unexpected results. It may be seen in Table II that the glycogenesis in the muscle does not change after ACTH treatment, but it decreases significantly after a single injection of growth hormone. No changes in glucose uptake were observed in either treatment.

TABLE I

Glucose Uptake and Glycogenesis by Diaphragms of Normal Rats Treated with Adenocorticotropic Hormone (ACTH) and Growth Hormone (GH)

Animals were treated with a single injection of 0.5 ml. hormone solution (5 mg.) 24 hr. before sacrifice

Experiment	Glucose uptake		Initial glycogen	Glycogenesis	
	Without insulin	With insulin		Without insulin	With insulin
Control	2.23 ± 0.13[a] (20)[b]	3.78 ± 0.37 (10)	3.95 ± 0.36 (20)	0.22 ± 0.09 (20)	1.52 ± 0.21 (10)
ACTH	2.43 ± 0.14 (6)	3.61 ± 0.10 (12)	3.55 ± 0.26 (18) $p = 0.3$[c]	-0.19 ± 0.17 (6) $p = 0.05$	0.54 ± 0.24 (12) $p < 0.01$
GH	2.14 ± 0.09 (12)	3.41 ± 0.05 (12)	2.64 ± 0.19 (24) $p < 0.01$	0.22 ± 0.16 (12)	0.44 ± 0.19 (12) $p < 0.01$

[a] mg. of glucose/g. of wet tissue/hr.; mean ± standard error.
[b] Number of animals in parenthesis.
[c] Fisher's p values.

TABLE II

Glucose Uptake and Glycogenesis by Diaphragms of Hypophysectomized Rats Treated with Adrenocorticotropic Hormone (ACTH) and Growth Hormone (GH)

Animals were treated with a single injection of 0.5 ml. hormone solution (5 mg.) 24 hr. before sacrifice

Experiment	Glucose uptake		Initial glycogen	Glycogenesis	
	Without insulin	With insulin		Without insulin	With insulin
Control	2.14 ± 0.14[a] (14)[b]	4.08 ± 0.17 (8)	3.99 ± 0.40 (16)	0.52 ± 0.11 (16)	1.66 ± 0.17 (10)
ACTH	1.92 ± 0.11 (18)	3.97 ± 0.12 (10)	4.39 ± 0.20 (28) $p = 0.3$[c]	0.17 ± 0.12 (18) $p = 0.05$	1.39 ± 0.21 (10)
GH	1.78 ± 0.28 (11) $p = 0.3$	3.52 ± 0.25 (12) $p = 0.1$	5.16 ± 0.26 (29) $p = 0.02$	-0.19 ± 0.14 (14) $p < 0.001$	0.29 ± 0.21 (15) $p < 0.001$

[a] mg. of glucose/g. of wet tissue/hr.; mean ± standard error.
[b] Number of animals in parenthesis.
[c] Fisher's p values.

In a recent report of Young *et al.* (2) it is clearly shown that pure growth hormone preparations do indeed produce glycosuria in normal cats.[1] Experiments reported herein are in agreement with the view that growth hormone is diabetogenic and that the diabetogenicity is at least in part due to its antagonistic action to insulin.

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[1] One of us (C. H. Li) wishes to thank Professor F. G. Young for many stimulating discussions during a recent visit to his laboratories.
[2] John Simon Guggenheim Memorial Foundation Fellow, 1949–1950.

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Hippuric Acid, Cinnamoylglucuronic Acid and Benzoylglucuronic Acid in the Urine of Normal Individuals and in Patients with Hepatic Dysfunction after Ingestion of Sodium Cinnamate

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It has long been known that, after ingestion of cinnamic acid ($C_6H_5CH:CHCOOH$), humans produce benzoic acid which is excreted in the urine as hippuric acid (1,2,3,4). Snapper, Yu and Chiang (5) have indicated that the cinnamic acid which escapes oxidation is probably excreted as monocinnamoylglucuronic acid. The presence of this compound became probable when a stoichiometric relationship was found between the amounts of glucuronic acid and cinnamic acid in the watery phase which separates from the ether after prolonged extraction of acidified urine. Cinnamoylglycine could not be discovered in the ether phase and it was shown that the amounts of free cinnamic acid excreted were negligible. These investigators did not follow up the possibility that not only cinnamoylglucuronic acid but also benzoylglucuronic acid was excreted after ingestion of sodium cinnamate.

To solve this point, the following experiment was performed:

After ingestion of benzoic acid the amount of benzoylglucuronic acid excreted in the urine can be estimated from the difference between the total benzoic acid and hippuric acid values. This method, first used by J. Neuberg (6), and later by Quick (7), gives satisfactory results because, after ingestion of sodium benzoate or benzoic acid, no appreciable quantities of free benzoic acid are excreted. Therefore, after ingestion of sodium cinnamate, the total benzoic acid present in the urine was compared with the amount of benzoic acid conjugated with glycine to form hippuric acid. If, in this experiment, the total benzoic acid were found to be the same as the benzoic acid present in the form of hippuric acid, then no benzoylglucuronic acid could be present in the urine. In

the course of this investigation it has become evident that simultaneous presence of cinnamic acid and of benzoic acid in the urine requires a modification of the calculations used for the determination of the total benzoic acid content of the urine.

Method

Fasting normal subjects and patients with cirrhosis of the liver ingested 6.9 g. of sodium cinnamate in gelatin capsules (equivalent to 5 g. benzoic acid). Breakfast was omitted. Lunch was allowed, but fruits and fruit juices were forbidden. Urine was collected 2, 4, and 6 hr. after the administration of the drug. The urine was kept in the icebox until used later in the day.

1. Determination of Conjugated Cinnamic Acid in the Urine

Conjugated cinnamic acid was determined by bromination after the urine was hydrolyzed. Quantitative determination of freshly precipitated cinnamic acid by bromine addition is readily possible if considerable excess of bromine is used (5). For the bromine addition an adaptation of the method described by Greenberg and Mackey was followed (8).

The bromine is produced by the reaction of bromate with an excess of bromide in acid solution. Then KI is added. The free bromine displaces the equivalent amount of iodine and the latter is titrated with $K_2S_2O_3$ as follows:

A 50 ml. aliquot of urine is placed in a beaker and 2 ml. conc. HCl are added. When the reaction is strongly acid (to Congo red paper), hippuric acid precipitates. The beaker is then left overnight in the icebox. The precipitate is filtered by suction and washed with 15 ml. of ice cold water. The filtrate is rendered alkaline by adding 10 ml. of 40% NaOH, and is boiled for 10 min. on a hot plate to hydrolyze the glucuronide. It is then cooled and acidified (until strongly acid to Congo red paper) by the addition of 10 ml. of conc. HCl. After cooling, the acid solution is extracted with chloroform for 3 hr. in a continuous extractor. When the extraction is terminated, the watery phase (upon testing with Congo red paper) must be acid. The chloroform is extracted by shaking with 2 portions of 15 ml. of 8% NaOH. The combined NaOH extracts are gently heated on a hot plate to evaporate the last traces of chloroform. Thereafter, the alkaline extracts are diluted to 50 ml. using volumetric flasks.

Reagents for bromine addition:

1. 0.01 *M* $KBrO_3$. 1.6701 g. of dried $KBrO_3$ dissolved in 1 l. of distilled water.
2. 50% KBr solution.
3. 20% KI solution.
4. 1% soluble starch.
5. 0.02 *N* $K_2S_2O_3$. Made up from 0.1 *N* thiosulfate before titration.

A 10 ml. aliquot is taken from the 50 ml. volumetric flask and placed in a 125 ml. Erlenmeyer flask. One ml. of 50% KBr is added to the flask, then 6 ml. of conc. HCl are added and the flask put in ice water. After cooling, 5 ml. of 0.01 *M* $KBrO_3$ are added dropwise. This solution is left for 10 min. in diffuse light. If the color remains

brown, titration can follow. If the solution becomes light yellow while standing, an extra 1 ml. of KBr and 5 ml. of $KBrO_3$ are added. After 10 min., 1 ml. of 20% KI is added. The flask is then placed in ice cold water until titration of the excess iodine with 0.02 N $Na_2S_2O_3$ or $K_2S_2O_3$. In the last phase of the titration, when the color has become a pale yellow, starch is added as indicator. The blank titration is carried out under the same conditions as the actual analysis with omission of the aliquot. The difference between the titration of the blank and of the urine samples gives the amount of bromine used up by the cinnamic acid. The thiosulfate does not need to be carefully standardized because the accuracy of the method depends on the bromate solution.

Recovery: (A) 25 ml. aliquot of fasting urine.
(B) 25 ml. aliquot of fasting urine plus 50 mg. cinnamic acid.
mg. cinnamic acid recovered: 47.5, 47.5, 47.1.

With this procedure a 95% recovery is attained. Bromine addition of one entire 2-hr. fasting specimen is equivalent to 44, 48, 23, 35, 31 mg. cinnamic acid. Average 36 mg.

The following equation simplified the calculation. Since 1 mole of thiosulfate is equivalent to 0.5 mole of cinnamic acid, 1 ml. of 0.02 N $K_2S_2O_3$ = 1.48 mg. cinnamic acid. The accuracy depends on the careful preparation of the bromate solution. The 5 ml. $M/100$ bromate is equivalent to 15 ml. 0.02 N thiosulfate. The thiosulfate solution is not exact and must be corrected by a blank. In the equation, it is assumed that a 10 ml. aliquot was taken from the 50 ml. volumetric flask (the first step in the bromine addition) and that 5 ml. of bromate was used.

$$(\text{blank}-\text{unknown})\frac{15}{\text{blank}}\times 5\times 1.48\times\frac{\text{total urine vol.}}{\text{urine aliquot}}\times\frac{100}{95}=\text{cinnamic acid in mg.}$$

2. *Determination of Total Benzoic Acid*

Total benzoic acid is determined by hydrolysis, followed by oxidation with HNO_3 and permanganate, chloroform extraction, and titration with sodium ethylate as described by Kingsbury and Swanson with slight modification (9). When tested by adding hippuric acid to samples of normal urine, 96% recovery was obtained. In

Hippuric acid added	Benzoic acid recovered[a]
g.	*g.*
1. 0.200	0.192
2. 0.200	0.192
3. 0.300	0.286
4. 0.293	0.282

[a] Expressed as hippuric acid. Blank of urine subtracted.

addition, we found that, if cinnamic acid was present, a small correction factor is necessary because traces of acid substances are formed from cinnamic acid during oxidation with HNO_3 and permanganate. These acidic substances are later dissolved by the chloroform during extraction. The following experiment shows that the presence of 25 mg. of cinnamic acid in the urine aliquot will increase the apparent value for total benzoic acid by 6.7 mg.

(A) 20 ml. urine
(B) 20 ml. urine plus 0.293 g. hippuric acid (= 0.200 g. benzoic acid)
(C) 20 ml. urine plus 0.293 g. hippuric acid plus 0.025 g. cinnamic acid

Titration with 0.119 *N* sodium ethylate: (A) 2.32 ml.; (B) 15.55 ml.; (C) 16.01 ml.
Calculation. Benzoic acid recovered, 0.192 g. Acid substances formed from cinnamic acid during oxidation and expressed as benzoic acid, 0.0067 g.

3. Determination of Hippuric Acid

This substance is determined by continuous ether extraction of duplicate aliquots of urine followed by hydrolysis, evaporation of the HCl, and formol titration of the glycine to pH 8.9 with the glass and calomel electrodes as previously detailed (9). 100% recovery is obtained by this method.

4. Glucuronic Acid

Glucuronic acid was determined by a qualitative method employing naphthoresorcinol (9) and the results tabulated by a four plus scale.

5. Presence of Cinnamoylglucuronic Acid after Ingestion of Cinnamates

Combined glucuronides can be separated from urine by lead acetate precipitation, followed by neutralization with NH_4OH and basic lead acetate precipitation (10). As with isolation of benzoylglucuronic acid, low temperatures and speed favor larger yields (11). The basic lead acetate precipitate is washed on the centrifuge with several portions of water. Lead is precipitated by H_2S, ice being added to the constantly stirred mixture. The PbS is removed by filtration, and the H_2S and NH_3 by a careful vacuum distillation. The remaining liquid is shaken with about 100 ml. ethyl ether to remove traces of hippuric acid. Aliquots of the aqueous phase were taken for bromine addition and for reduction by the Shaffer-Hartmann method.

DISCUSSION

After oral administration of 6.9 g. of sodium cinnamate, normal persons exhibit an increased excretion of glucuronides (Table I). The amounts of benzoic acid not combined with glycine are negligible, varying from 0 to 0.2% of the total benzoic acid excreted. These results indicate that all of the benzoic acid obtained from the oxidation of cinnamate was conjugated with glycine to form hippuric acid. As a consequence, the formation of benzoylglucuronic acid can be excluded. At the same time, a cinnamic acid compound totaling over 0.5 g. was excreted. The following suggestive data lend support to the observations of Snapper, Yu and Chiang (5) that this is monocinnamoylglucuronic acid.

TABLE I

Glucuronides Produced after Ingestion of 6.9 g. of Sodium Cinnamate in Normals and in Patients with Liver Disturbances

	Glucuronides	Cinnamic acid[a]	Total benzoic acid[b]	(A) Corrected Total benzoic acid[c]	(B) Benzoic acid combined with glycine	(A)−(B) Non-hippuric benzoic acid	
		g.	g.	g.	g.	g.	per cent A
Normals							
1. 2 hr.	++	0.079	1.54	1.52	1.55	−0.03	0
4 hr.	−						
6 hr.	−						
2. 2 hr.	++	0.215	1.68	1.63	1.67	−0.04	0
4 hr.	++++						
6 hr.	+						
3. 2 hr.	++++	0.429	2.31	2.20	2.41	−0.21	
4 hr.	+++	0.272	1.88	1.81	1.81	0.	
6 hr.	++	0.102	0.48	0.46	0.48	−0.02	
Total		0.803	4.67	4.47	4.70	−0.23	0
4. 2 hr.	+	0.045	0.98	0.97	0.97	0	
4 hr.	+++	0.223	2.08	2.02	1.93	0.09	
6 hr.	++	0.104	1.10	1.07	1.06	0.01	
Total		0.372	4.16	4.06	3.96	0.10	0.2
5. 2 hr.	+++						
4 hr.	+++						
Total		0.442	3.57	3.45	3.54	−0.09	0
6.							
6 hr. total	++++	0.756	4.81	4.61	4.82	−0.21	0
7.							
6 hr. total	++++	0.577	4.54	4.39	4.56	−0.17	0
Cirrhosis							
1. 2 hr.	+++						
4 hr.	++++						
6 hr.	+						
Total		1.054	3.71	3.43	3.42	0.01	0.3
2. 2 hr.	−						
4 hr.	+						
6 hr.	++++						
Total		0.447	3.34	3.22	3.17	0.05	1.6
3. 2 hr.	+						
4 hr.	++++						
6 hr.	++++						
Total		0.809	2.77	2.55	2.19	0.36	14.1

[a] Chloroform extraction, bromine addition; 5% added, blank subtracted (see text).
[b] 4% added (see text).
[c] Corrected for acid substances formed from oxidation of cinnamic acid compounds.

Since, after ingestion of sodium cinnamate by a normal individual, all the gulcuronic acid present in the urine is conjugated with cinnamic acid, for each molecule of the latter substance one molecule of glucuronic acid must be present.

Samples of urine were treated with neutral and basic lead acetate, the lead removed (see Method, paragraph 5), and cinnamic acid and reducing power of the aqueous phase were determined. The amounts of cinnamoylglucuronic acid calculated from the quantity of cinnamic acid was the same as the value calculated from the quantity of glucuronic acid found.

Samples of Urine from Two Normal Subjects Who Had Ingested Sodium Cinnamate

Subject	Cinnamoylglucuronic acid (g.)[a] Bromine addition	Reduction
1	0.069	0.066
2	0.042	0.045

[a] Calculated as monocinnamoylglucuronic acid.

The qualitative tests show that not only the normal controls but also the patients with liver damage produce glucuronides under these circumstances. In the oral sodium benzoate test of Quick (12), which uses benzoate in a dose equivalent to the cinnamate given in this investigation, the production of 3 g. of benzoic acid conjugated with glycine in 4 hr. is taken as normal. In two of the liver cirrhosis patients who, after ingestion of sodium cinnamate, showed a hippuric acid excretion not far from normal, the percentages of benzoic acid not combined with glycine were 0.3 and 1.6%, respectively. Thus, the glucuronides excreted by these patients were conjugated, as in the normal subject, with cinnamic acid. In the third patient, who had more severe liver damage, only 2.19 g. of benzoic acid was combined with glycine and, at the same time, it can be noted that 14.1% of the benzoic acid was not in combination with glycine. For the methods employed, this value is significant. As no free benzoic acid is excreted (6,7), these findings are indirect evidence that this patient with severe liver damage excreted, after ingestion of sodium cinnamate, not only cinnamoylglucuronic acid but also benzoylglucuronic acid (9,14).

Thus, in the cirrhotic liver, impairment of the glycine conjugation can become so severe that even the relatively small quantity of benzoic acid formed after oxidation of the cinnamic acid cannot be conjugated to hippuric acid. Under these circumstances, part of the benzoic acid formed is excreted as benzoylgucuronic acid.

Whereas the normal individual excretes only hippuric acid and cinnamoylglucuronic acid after ingestion of 6.9 g. of sodium cinnamate, the patient with severe liver damage excretes not only these two conjugation products but also benzoylglucuronic acid. It follows that different excretion patterns are possible, and the various pathways in the metabolism of cinnamic acid as seen from this study are presented in Fig. 1 in schematic form.

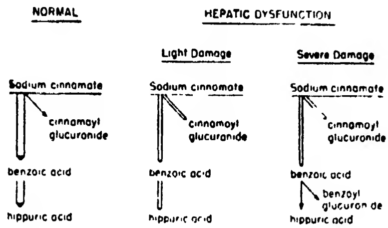

FIG. 1. Metabolism of cinnamic acid.

SUMMARY

A method for the quantitative estimation of cinnamic acid in urine by hydrolysis, chloroform extraction, and bromine addition is given. When cinnamic acid was added to urine samples, a 95% recovery was obtained.

After ingestion of 6.9 g. sodium cinnamate, normal subjects and patients with cirrhosis of the liver excrete considerable amounts of glucuronides in the urine. Normal subjects oxidize the main part of the cinnamate to benzoate and excrete it as hippuric acid. The remaining cinnamate is conjugated with glucorunic acid. In normals, all of the benzoic acid is excreted as hippuric acid and the formation of benzoylglucuronic acid can be excluded.

Subjects with severe liver disturbances (1) oxidize cinnamate at a diminished rate; (2) in one subject the amount of benzoate produced exceeded the capacity of the disturbed glycine mechanism with the result that some was conjugated as benzoylglucuronic acid. The presence of the latter was shown by the difference between the values for total benzoic acid and benzoic acid bound with glycine.

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The Proteins of Green Leaves. II. Purine, Pentose, Total Phosphorus and Acid-Labile Phosphorus of the Cytoplasmic Proteins of Spinach Leaves [1]

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Introduction

Evidence has been previously presented (12) that the cytoplasmic proteins of spinach leaves consist of 2 protein components, Fraction I and Fraction II, which can be distinguished by electrophoresis and can be separated by precipitation with $(NH_4)_2SO_4$. Fraction I, which is the subject of this investigation, has the following properties: (1) It is the main protein component of spinach cytoplasm, making up about 75% of the total cytoplasmic proteins; (2) The protein is electrophoretically homogeneous, neither the mobility of the protein, nor the shape of the Tiselius scanning pattern, changing upon repeated precipitation of the protein with salt; (3) The protein is a phosphatase enzyme in that it can hydrolyze glycerophosphate, adenosine triphosphate (ATP), creatine phosphate, *etc.*; (4) Nearly all of the bound auxin in spinach leaves is found in this protein.

The work to be described below will show that, in addition to the properties listed above, Fraction I protein contains, or has closely associated with it, substantial amounts of purine, pentose and phosphorus. The phosphorus relationship is particularly interesting, since about 25% of the total P of the protein is easily hydrolyzable to orthophosphate in acid solution under conditions similar to those required to hydrolyze high-energy P. Thus, this acid-labile P bound to protein is present in a form such that it is not extracted by the methods so frequently used for extracting ATP, *etc.*, from animal tissues.

[1] This work was supported by a grant from the American Cancer Society. We are indebted to Miss Martha Kent for the microbiological assays.

There is relatively little modern information on the P metabolism of higher plants. Albaum and Umbreit (1) extracted etiolated oat embryos at various stages of their development with cold trichloroacetic acid (TCA). They found that only 60% of the total P of young tissues was soluble in TCA after prolonged extraction periods, and only 83% from older tissues. No attempt was made to characterize the nature of the acid-insoluble P. Similarly, Emerson, Stauffer and Umbreit (5) found that only 45% of the total P contained in *Chlorella* was soluble in TCA after a 24 hr. extraction period. Thus, in the two tissues so far examined, it is evident that much of the phosphorus of plant cells is not removed by procedures which have been considered adequate for the study of P metabolism in animal tissues.

Method and Materials

Preparation of Fraction I Protein

The bulk protein of cytoplasm was prepared as described previously (12) from spinach leaf *Whole Cytoplasm* by a single precipitation with 0.38 saturated, neutral $(NH_4)_2SO_4$. The protein was completely redissolved in glass-distilled water, there being enough salt carried along with the protein precipitate to maintain the protein in solution when redissolved in water. The protein was preserved for later use by dividing the solution into 2.0 ml. lots, immediately freezing with the aid of a dry ice-cellosolve bath, and storing at − 15°C. until used.

Several improvements have been incorporated into the original fractionation scheme developed for spinach leaf protoplasm. By using an Eppenbach colloid mill, the preblending originally required is avoided. For the experiments to be described in this paper, greenhouse grown spinach leaves were chopped into approximaetly $cm.^2$ pieces and passed directly into the colloid mill. The concentrated, basket-centrifuged juice was frozen without delay in lyophil flasks in a dry ice cellosolve mixture and held in a deep freeze until the *Whole Protoplasm* was dried by lyophil. The lyophil-dry material was stored in the dark *in vacuo* over P_2O_5 until used for the preparation of *Whole Cytoplasm* and Fraction I protein. Usually, several kg. of spinach leaves were worked up into *Whole Protoplasm* which was stored as described and used in small quantities for the preparation of Fraction I protein. A typical preparation is the following. Forty g. of whole protoplasm were dispersed into 175 ml. of glass-distilled water at 0°C. The dispersion was centrifuged cold for 45 min. at 20,000 *g* in a Sorvall high speed centrifuge to remove most of the chloroplastic material. Since the supernatant was still quite green, ice-cold, neutral, saturated $(NH_4)_2SO_4$ was added until 0.25 saturation was attained. When sufficient salt was present to prevent freezing, the temperature of the solution was lowered to − 5°C. and kept at this temperature throughout the rest of the manipulations. After addition of salt to the green supernatant, all of the green material could be removed by centrifuging at 20,000 *g* for 30 min. Enough more $(NH_4)_2SO_4$ was added to the clear brown supernatant to give a 0.38 saturated solution, and Fraction I protein removed by centrifuging 15 min. at 6000 *g*. The

precipitated protein was immediately redissolved in 50 ml. of ice-cold glass-distilled water, divided into 2.0 ml. lots and quick-frozen. By this procedure, 40,0 g. of *Whole Protoplasm* produced from 1 kg. of fresh spinach leaves yielded 4.1 g. of Fraction I protein. Not all of the Fraction I protein contained in the preparation of whole cytoplasmic proteins is precipitated by this procedure, since we have purposely chosen a concentration of $(NH_4)_2SO_4$ such that the precipitate of Fraction I protein will not be contaminated by Fraction II proteins.

Phosphorus Determinations

Total P was determined after complete digestion of a sample with conc. H_2SO_4 for 16 hr. at 110°C. To clear the digestion mixture completely, 1–3 drops of 30% H_2O_2 were added and the excess peroxide removed by continued heating of the sample at 110°C. for 2 hr. The clear solution was then diluted with water and heated at 100°C. for 10 min. to hydrolyze pyrophosphates. Phosphorus was determined by the method of Berenblum and Chain (2). The stable blue color was read in a Klett-Summerson colorimeter. Blank determinations of the entire procedure without P gave negligible colorimeter readings.

"Apparent" inorganic P was determined by calcium precipitation according to the method of Umbreit *et al.* (11). However, such precipitates generally carried along with them sufficient organic matter to interfere with the P determinations. The calcium precipitates, therefore, were also frequently subjected to complete digestion before analysis for P.

Purine Determinations

Use was made of the microbiological assay developed by Mitchell and Houlahan (9), using an adenineless mutant of *Neurospora*. Six concentrations of adenine were used for calibrating the bioassay. Unknown samples were hydrolyzed for 2 hr. in *N* HCl at 100°C., and then neutralized before microbiological analysis. This assay has been considered specific for adenine and hypoxanthine, although the mold also responds to guanine (8).

Pentose Determinations

The orcinol-HCl method for pentose described by Umbreit *et al.* (11) was used. The green color complex has an absorption peak at 675 mμ and the color of the solutions was read in a Coleman Junior spectrophotometer at this wavelength. Ribose was used as a standard, although arabinose gave identical values.

Experimental Results

Total Phosphorus Content of Fraction I Protein

Undenatured Fraction I protein prepared by $(NH_4)_2SO_4$ precipitation contains 0.3–0.5% total P. Since approximately 10% of the dry weight of spinach leaves consists of Fraction I protein and dry leaves contain about 0.5% total P, it is evident that about 6% of the total leaf P is directly associated with the bulk cytoplasmic protein. As will be seen from later experiments, this value undoubtedly represents a minimum figure.

Acid-Labile Character of the Phosphorus Associated with Fraction I Protein

When the protein was suspended in 1 *N* HCl at 0°C., a portion of its P content was released as apparent inorganic P. If the protein was heated for a brief period in 1*N* HCl, the amount of apparent inorganic P released was increased. Heating for 7 min. was sufficient to double the amount of calcium-precipitable P. Thus, it appeared that some of the P associated with the protein was in labile combination. The following experiments will show that it is possible to remove most of the P from the protein as two distinct subfractions. One is soluble in cold acid and appears to contain only inorganic P; the other P-containing material is insoluble in cold acid, but can be released by heating the protein for 2 min. in hot acid, and it is the latter material which contains labile P.

The Extraction of Phosphorus from Fraction I Protein

Solutions containing 11.3 mg. of the main cytoplasmic protein were precipitated and irreversibly denatured by 5.0 ml. of 1 *N* ice-cold trichloroacetic acid (TCA). The precipitate was spun down, and after decanting, the clear supernatant solution was evaporated to a small volume, totally digested with H_2SO_4 and analyzed for P. As shown by the data of Table I, 53.5% of the total P associated with the protein was removed by cold acid. The precipitate was extracted again in exactly the same manner.

TABLE I

Distribution of P in Cold and Hot Acid Extracts Obtained from Fraction I Protein of Spinach Leaf Cytoplasm

11.3 mg. of protein used for distribution analysis

Treatment of protein	γ P/mg. protein	Per cent total protein P
None	5.04	100
1st cold TCA extract	2.7	53.5
2nd cold TCA extract	0.23	4.6
2 min. hot TCA extract	1.63	32.3
Protein remaining after cold and hot TCA extraction	0.32	6.3

In contrast to the first extraction, the second cold acid extract contained only 8.6% as much P (4.6% of total P) as found in the first extract. Evidently, only about 60% of the total P of the protein is easily removed with cold acid. Other experiments have shown that extending the extraction period from 5 min. to 8 hr. did not produce a significant increase in the amount of P removed by cold acid, and that HCl, H_2SO_4, and TCA were equally effective as cold acid extractants.

Although 40% of the total P associated with undenatured Fraction I protein appeared to be "bound" to the protein so that it was not removed with cold acid, nevertheless, most of the bound P could be released from the protein into a clear extract by a simple procedure.

The cold acid-extracted protein was resuspended in 5.0 ml. of cold 1 *N* TCA, heated in a boiling water bath for 2 min. with vigorous stirring, quickly cooled to 0°C. in an ice bath, and the protein precipitate spun down. The clear supernatant solution was analyzed for total P after digestion. Similarly, the protein precipitate was also analyzed for P after digestion. The data in Table I show that the 2 min. hot acid treatment released 32.3% of the total P (83.5% of the P remaining on the protein precipitate after 2 extractions with cold acid) leaving a protein precipitate nearly free of P. Thus, it is possible to account for about 96% of the total P associated with the bulk cytoplasmic protein by two extractions with acid. Calculation reveals that about 3% of the total P contained in spinach leaves is "bound" to Fraction I protein of cytoplasm.

TABLE II

The Release of P from Fraction I Protein by Heating in 1 N Acid as Determined by Subsequent Total P Analysis of the Protein Precipitate and Supernatant Solution

Time of heating *min.*	Total P in protein precipitate *γ/mg.*	Total P in supernatant solution *γ/mg.*
0	2.5	0.18
1	0.7	1.96
2	0.43	2.14
4	0.43	2.14
8	0.43	2.14
64	0.36	2.18

A 2 min. heating period in acid releases as much of the protein-bound P into a clear supernatant solution as more prolonged heating periods as shown by Table II. Equal samples of protein were first extracted with cold TCA, the precipitates spun down and the supernatant solutions discarded. The protein precipitates were resuspended in cold acid and then heated in a boiling bath for the times indicated in Table II, rapidly chilled to 0°C. in an ice bath, the precipitates spun down, and both the supernatant solutions and the precipitates analyzed for total P after digestion. Table II shows that only a slight increase in the amount of total P released from the protein occurred with a heating time of 64 min. compared to 2 min.

Instead of digesting the material released into the supernatant solution by the 2 min. hot acid treatment as in Table II, analysis was made for apparent inorganic P in this extract by calcium precipitation.

Only 0.2 γ P/mg. of protein were precipitated compared to the 2.14 γ P/mg. known to be present as the result of total digestion. However, when the 2 min. hot acid protein-free extract was heated additionally for 5 min. (total heating time in acid, 7 min.) 0.8 γ P/mg. were precipitated with calcium. Two conclusions can be reached on the basis of these data and that of Table II: (1) nearly all of the P bound to Fraction I protein can be released into a non-protein extract by heating the protein for 2 min. in 1 *N* acid. (2) The P is not released from the protein as inorganic P, but in some form from which it can be subsequently released as orthophosphate by additional heating in 1 *N* acid. The following experiments were carried out in an attempt to characterize the nature of the P associated with the bulk cytoplasmic protein.

Preparation of the P-Containing Materials by Cold and Hot Acid Extraction

To facilitate analysis, rather large scale preparations of the materials soluble in cold acid and those released by the 2 min. hot acid extract were made, following in complete detail the scheme already outlined except that 1 *N* HCl was substituted for TCA. Immediately after centrifuging down the protein, the two extracts were frozen and dried by lyophil thus freeing them of HCl. Drying was continued for 48 hr. *in vacuo* over P_2O_5. The dry samples were used for the analyses described below. Most of the dry extracts could be directly scraped out of the lyophil flasks although both materials are sufficiently hygroscopic to make handling difficult.

Properties of the P-Containing Materials Found in the Cold Acid Extract

The material contained in this extract behaved as an indicator; it was nearly colorless in acid solutions and yellow-brown in basic solutions. As mentioned previously, none of the P found in this fraction appeared to be of a labile character. Experiments on 2 different preparations showed that an average of 83.3% of the total P was precipitated by calcium. Heating the preparation for 30 min. in 1 *N* HCl at 100°C. did not increase the amount of P which could be precipitated by calcium. Inorganic P added to the sample was quantitatively recovered by calcium precipitation.

All of the P contained in the dry material obtained by cold acid extraction is soluble in hot 80% alcohol, although there is a non-P-containing residue which does not dissolve. Fig. 1 is an absorption spectrum of the alcohol extract which reveals two distinct maxima, one at 260 mμ, and another at 330 mμ. The sharpness of the peak at 260 mμ

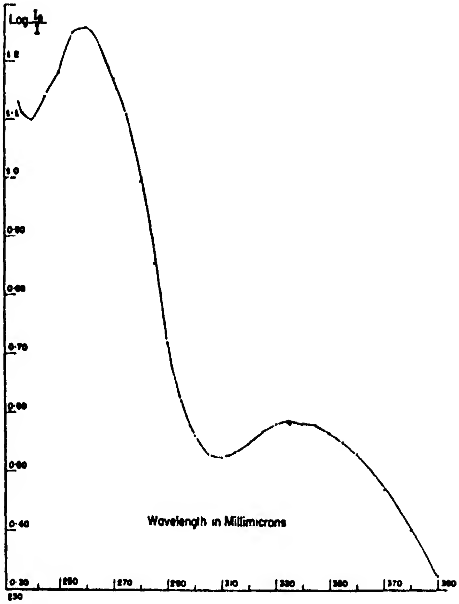

FIG. 1. Ultraviolet absorption spectrum of the material contained in the cold acid extract of Fraction I protein. After drying, the material was redissolved in 80% ethyl alcohol. Approximately 0.2 mg. dry material/ml.

suggests the presence of purines (10) and, as a matter of fact, the presence of purine has also been demonstrated by microbiological assay. The results are shown in Table III.

The significance of the second absorption maximum at 330 mμ is obscure, but it was noted that the peak disappears without effect on the 260 mμ maximum, when the extract was previously heated with 1 *N*

TABLE III

Distribution of Phosphorus, Purine, and Pentose Associated with Fraction I Protein

Material analyzed	γ/mg. Fraction I protein		
	Phosphorus	Purine	Pentose
Whole protein	3.4	6.7	15.7
Cold acid extract	1.7	3.3	10.3
Hot acid extract	1.5	3.9	5.5
Final protein precipitate	0.08	0	—

HCl for 2 hr. at 100°C. This may indicate either the destruction of an impurity or hydrolysis of a compound with consequent alteration of the resonating structure.

The cold acid extract contains some material which partially inhibits the *Neurospora* microbiological assay for purines. Small amounts of cold acid extract definitely caused the mutant to grow, indicating the certain presence of purines, but the growth was small and did not increase proportionally to increasing concentrations of the cold acid extract.

Pentose determinations by the orcinol method were not completely satisfactory because of the fact that a brown color developed instead of the green color characteristic of the reaction with pure pentose in the presence of the materials extracted by cold acid. However, there was a definite absorption maximum detected at 675 mμ. It was possible to recover about 75% of ribose added to the cold acid extract before reaction with orcinol—HCl, showing that the brown color only partially obscured the maximum at 675 mμ. However, it is necessary to consider both the purine and pentose values listed in Table III as provisional.

The fact that the total P in the cold acid extract is soluble in 80% EtOH seemed initially to be inconsistent with the solubility properties of orthophosphate, particularly since as much as 50% of the total P can be dissolved in hot absolute EtOH. However, when inorganic P was added to the cold acid extract before acidification and drying by lyophil, the added P was quantitatively recovered in a hot absolute EtOH extract. Evidently, at these levels of P, solubility in a dry organic solvent is useless as a method of distinguishing between inorganic and organic P compounds.

The yield of the material in the cold acid extract is a difficult matter to assess. Values averaging around 35% of the weight of the protein have been obtained by drying the HCl extract. However, such material is contaminated by $(NH_4)_2SO_4$ carried along with the protein precipitate during the preparation of Fraction I protein.

Properties of the Materials Found in the Hot Acid Extract

Fig. 2 is a hydrolysis curve illustrating the labile nature of the P contained in the hot acid extract. After preparation by the 2 min. hot acid procedure, small amounts of the dry extract ("R" in Fig. 2) were redissolved in ice cold 1 *N* HCl and additionally heated for various

lengths of time at 100°C., the solutions immediately chilled and the released inorganic P precipitated by calcium in the cold. The calcium precipitate was analyzed for total P. Fig. 2 shows that 54.5% of the labile P is released in 7 min., 94% in 16 min. and all in 32 min. since there was no further increase when the sample was heated 64 min. That a small amount of inorganic P should be found before heating is understandable since it was necessary to heat the protein for 2 min. in order to release the P-containing material from Fraction I protein with the probability that a small amount of the acid-labile P was released as inorganic P during this process.

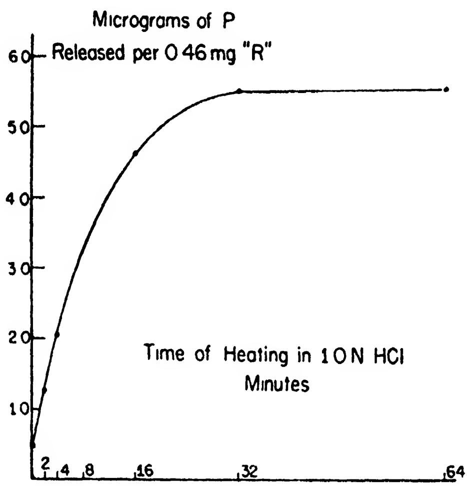

FIG. 2. The release of phosphorus as a function of time of heating in 1 N HCl from the material obtained by hot acid extraction of Fraction I protein.

Total P analysis showed this sample to contain 11.2 γ P/0.46 mg. dry extract. As shown in the curve, 5.5 γ P/0.46 mg. dry extract was released by heating in 1 N HCl for 32 min. Hence, the ratio of acid-labile P to total P is 1:2. The experiment has been repeated with similar results using a different batch of spinach leaves.

The presence of purines in the hot acid extract is suggested by the absorption spectrum illustrated by Fig. 3. There is a distinct maximum apparent at 260 mμ when the hot acid extract is dissolved in neutral buffer. The peak, however, is broader than that exhibited by pure adenine (10), and the small irregularity at 265 mμ may indicate the presence of other purines or pyrimidines. In contrast to the cold acid extract there is no distinct maximum at 330 mμ in the hot acid extract.

The presence of purine was confirmed by *Neurospora* microbiological assay and the results are given in Table III. By the orcinol method, the hot acid extract was also shown to contain pentose. In contrast to the cold acid extract only slight interference was noted with the adenine assay and the pentose determination of the hot acid extract.

The material contained in the hot acid extract makes up about 10% of the weight of Fraction I protein. The amounts of purine, pentose and P found in the starting protein, cold and hot acid extracts, and the protein precipitate remaining after acid extraction are given in Table III, where they are computed on the basis of the amount of protein present before extraction.

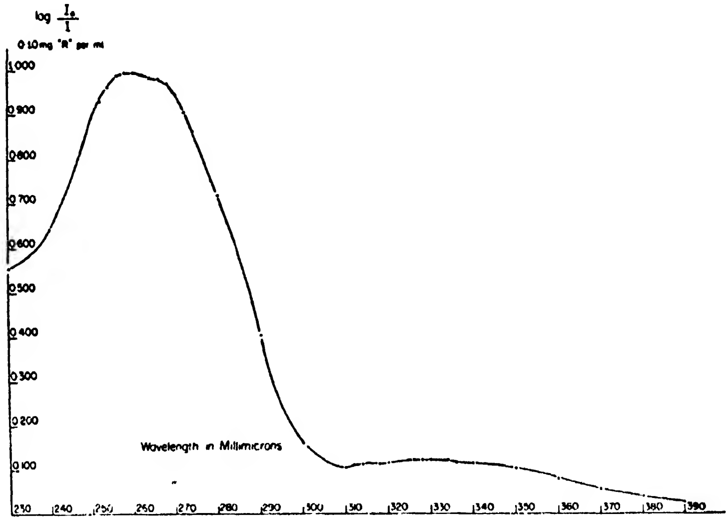

FIG. 3. Ultraviolet absorption spectrum of the material contained in the hot acid extract of Fraction I protein. After drying, the material was redissolved in neutral buffer.

Less than 20% of the total P in the hot acid extract, prepared as described, can be precipitated with calcium, indicating that at least 80% of the P is combined with organic material. Barium acetate at pH 6.8 (11) precipitated less than 40% of the total P. No P was precipitated with $Ba(NO_3)_2$ at pH 2.5 within 24 hr., thus showing the absence of metaphosphate (6). In view of the exact 1:1 ratio of acid-labile to acid-stable P, it is not improbable that the acid-labile P bound to Fraction I is of the pyrophosphate type. The possiblity that the P-

containing material in the hot acid extract is similar to yeast adenylic acid needs to be considered. While it is true that the P contained in adenosine-3-phosphate is labile in hot 1 N acid, nevertheless, the extent of hydrolysis is much greater than that experienced with the material in the hot acid extract. Levene and Bass (7) found the P in yeast adenylic acid to be 72.8% hydrolyzed in 10 min. and 88% in 60 min. The material described here is only 34% hydrolyzed in 10 min. and 50% in 32 and 60 min. Therefore, the resemblance between the material in the hot acid extract and yeast adenylic acid is not immediately apparent.

Only about 16% of the weight of hot acid extract can be accounted for as purine, pentose, and P. Therefore, it cannot be said with assurance that the organic P is combined with pentose and purine, although this possibility is suggested by the approximate molar ratio of 1 purine: 1 pentose: 2 P.

Absence of Appreciable Acid-Labile P in the Chloroplasts

Precipitated and washed grana obtained from spinach leaves contain very little labile P. Two 10 mg. samples of lyophilized chloroplasts were extracted twice with 5 ml. portions of cold TCA. The extracts were discarded. The chloroplast precipitate was resuspended in cold TCA; one sample was kept cold while the other was heated for 7 min. at 100°C. After spinning down the precipitates, the extracts were analyzed for apparent inorganic P. The unheated extract contained 0.007 γ P/mg. chloroplasts, while the heated sample contained 0.08 γ P.

It is perhaps possible that the trace of labile P present may have resulted from contamination of the grana with cytoplasm (12). It is, therefore, evident that the easily detectable acid-labile P of the spinach leaf is confined to the cytoplasm where most of it appears to be bound to Fraction I protein.

Bound P and Storage of Fraction I Protein

The P initially bound to Fraction I protein is slowly released when the protein is allowed to stand in solution, even at 2°C., and no longer precipitates with the protein in the presence of cold TCA. A sample of Fraction I which, after cold TCA extraction, contained 8.84 γ of acid-labile P, contained 1.4 γ 4 days later and none after standing 5 days in neutral solution at 2°C. Since both the labile and the stable P are lost from the protein by this treatment, it must be concluded that all of the material obtained initially as a hot acid extract is changed by

standing so that it no longer precipitates with the protein in acid solutions. It is not certain whether the liberation of this material is also accompanied by hydrolysis of the labile P to inorganic P. Irrespective of the proper explanation, it is now our practice to subdivide the protein solution into 2.0 ml. lots immediately after preparation, and then freeze the samples in a dry-ice cellosolve bath and store in a deep freeze at − 15°C. until used. In this way, the protein can be kept indefinitely without loss in bound labile P.

Purine, Pentose and Phosphorus Content of Whole Cytoplasm

Each time Fraction I protein is precipitated with neutral $(NH_4)_2SO_4$, there is a loss in TCA-precipitable acid-labile P. Fraction II proteins do not contain significant amounts of P. It was of interest, therefore, to examine whole cytoplasm for purine and P in order to get some idea of how much acid-labile is bound to the bulk protein before precipitation.

TABLE IV

Loss in Phosphorus and Purine From Fraction I Protein as a Result of Precipitation with $(NH_4)_2SO_4$

Protoplasmic fraction	Subfraction	γ per mg. protein	
		Phosphorus	Purine
Whole cytoplasm	Cold acid extract	—	12.0
Whole cytoplasm	Hot acid extract	2.28	10.0
Whole cytoplasm	Final protein precipitate	0.5	0
Fraction I protein	Cold acid extract	2.0	3.9
Fraction I protein	Hot acid extract	0.92	3.9
Fraction I protein	Final protein precipitate	0.06	0

After removal of chloroplastic material by high speed centrifugation (12), whole cytoplasm was subjected to the P fractionation scheme described previously. The data are presented in Table IV. The cold acid extract obtained from whole cytoplasm is a conglomeration of all of the acid-soluble forms of P in the cell and consequently lacks meaning in relation to the proteins of the cytoplasm. It is noteworthy, however, that the cold acid extract of whole cytoplasm contains large amounts of purine. Also, there is little P of an acid-labile nature contained in this extract.

There is 2.5 times more P in the cold TCA protein precipitate of whole cytoplasm than in a similar TCA precipitate of Fraction I protein prepared from the same sample of whole cytoplasm. There is also 2.6 times more purine in the TCA precipitate of whole cytoplasm than in a similar precipitate of Fraction I protein. Since the loss of P cannot be accounted for in the Fraction II protein it can be concluded that some of the material containing acid-labile P is released when the Fraction I protein is precipitated with $(NH_4)_2SO_4$, particularly since the ratio of purine: P remains the same in both whole cytoplasm and Fraction I protein. Further, most if not all of the TCA-precipitable, acid-labile P in whole cytoplasm is associated with the bulk protein which is also a phosphatase enzyme. Calculation shows that 2.3% of the total leaf P is acid-labile P bound to Fraction I protein of cytoplasm.

Phosphorus Distribution in Whole Lyophilized Spinach Leaves

The distribution of P in relatively undisturbed spinach leaves was investigated. Fresh leaves were almost instantaneously frozen in liquid air in order to immobilize the labile tissue constituents, and then dried by lyophil. After grinding to 40 mesh, 5.0 mg. samples of dry leaf powder were analyzed for P distribution according to the scheme described previously. Since whole leaves do not sediment satisfactorily in a centrifuge, extraction and filtration was performed on a sintered glass funnel. As shown by the data in Table V, only 60% of the total leaf P

TABLE V

Phosphorus Distribution in Whole Lyophilized Spinach Leaves

Material analyzed	γ Total P/mg. dry wt.	Per cent total P
Whole tissue	4.98	100
Cold acid extract	2.98	60.0
Hot acid extract	1.50	28.8
Final protein precipitate	0.69	13.5

is soluble in cold acid, a result which parallels previous work on oat embryos and *Chlorella* (5). A second extraction with cold acid removed less than 8% of the amount of P obtained in the first extraction. If the cold acid-extracted precipitate is heated for 2 min. in 1 *N* HCl at 100°C., 28.8% of the total P is released from tissue constituents and appears in the clear acid extract. After the hot acid treatment, only 13.5% of the total P still remains bound to tissue. Other experiments have shown

that, if the heating time is extended beyond 2 min., nearly all of the P is released.

The acid-labile character of the P in the hot acid extract obtained from whole leaves is shown by the following data. Whole, lyophilized leaves were extracted twice with cold TCA and the extracts discarded. The tissue was heated with 5 ml. of 1 *N* HCl for 2 min. in a boiling water bath, immediately cooled to 0°C., and the tissue removed by filtration. The clear extract was analyzed for total P, apparent inorganic P, and apparent inorganic P after additionally heating the extract. The initial 2 min. heating period produced 0.4 γ inorganic P/10 mg. tissue. Heating 5 min. more produced 3.1 γ inorganic P/10 mg.; 30 min. caused the release of 10.3 γ/10 mg., which is taken to represent the total acid-labile P in the hot acid extract. Thus, 34% of the labile P is released as inorganic P by a total heating time of 7 min. Complete digestion of the material in the hot acid extract showed it to contain 20.6 γ total P/10 mg. Therefore, the ratio of labile P to stable P is 1:1. Evidently, 15% of the total P contained in spinach leaves is acid-labile P bound to tissue constituents.

Analysis of the hot acid extract prepared from the same batch of spinach leaves showed the presence of 60 γ of purine/10 mg. of leaves. Thus the molar ratio of purine (as adenine) to total P is 1:2. It should be noted that 0.6% of the dry weight of spinach leaves is found in the hot acid extract as purine.

Discussion

On the basis of Chibnall's classic work on spinach cytoplasmic proteins (4), it has long been thought that the leaf proteins contain only minute amounts of purines and relatively little P. However, the fact that adenine has been shown to be a growth factor for the expansion of some leaves (3) suggests that leaves might be a relatively rich source of purines. Chibnall's purest preparation of cytoplasmic protein contained only 0.13% P," . . .but deliberate search for the nuclear bases has always given a negative result" (4). From the experiments on spinach leaves presented here, it is evident that cytoplasmic proteins are much richer sources of purines and of P than had been previously thought. Even by neglecting the purine and P found in the cold TCA extract of whole cytoplasm, it can be seen that enough purine precipitates with the proteins to account for 1% of the weight of the proteins. If it is considered that the cold acid-extractable purine is also an intimate con-

stituent of the cytoplasmic proteins, then 2.2% of the weight of the cytoplasmic proteins consists of purine. Similarly, the amount of P left on the proteins after cold acid extraction amounts to as much as 0.3% of the weight of the cytoplasmic proteins.

To account for the discrepancy in purine and P values presented here and those reported previously, it is necessary to consider the methods used by others to prepare cytoplasmic proteins. Chibnall (4) precipitated the proteins isoelectrically at a pH of about 4. However, this treatment results in almost complete removal of bound P from the protein. Since the P is probably bound to a material containing purine and pentose, it can be inferred that such a treatment also results in complete removal of purine.

Summary

1. Fraction I protein, which is the main protein component of spinach leaf cytoplasm and at the same time a phosphatase enzyme, contains approximately 0.3–0.5% total P. Of the protein P, 25% is acid-labile but is not removed by cold acid extraction procedures customarily used in P metabolism studies.

2. After release from the Fraction I protein, the P is contained in 2 distinctly different subfractions, one of which is removed from the protein by cold acid extraction; the second is only removed after heating the protein at 100°C. for 2 min. in 1 *N* acid. The labile P of the protein is confined to the latter extract.

3. One-half of the total P of the hot acid subfraction is labile P and is completely released as orthophosphate by heating for 32 min. The remainder is stable and is not detected as orthophosphate until total digestion.

4. No significant amounts of acid-labile P are found in the chloroplasts.

5. The combination of protein with the P-containing materials is easily broken by allowing the protein to stand in solution even at 0°C.

6. Phosphorus distribution studies on lyophil-dry spinach leaves show that only 60% of the total leaf P is extracted with cold acid. Heating the tissue for 2 min. in hot acid releases most of the remainder of the P not extracted with cold acid. The P bound to tissue constituents and released by hot acid is shown to be acid-labile, in the ratio of 1 labile P:1 stable P. Of the total P contained in leaves, 14% is acid-labile P bound to tissue constituents.

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The Basic Amino Acid Contents of Collagen and of Deaminized Collagen [1]

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Introduction

The most recent and reliable figures for the basic amino acid content of collagen are those of Macpherson (1) for arginine, histidine, and lysine, and of Rees (2) for hydroxylysine. These results were presumably obtained on the same sample of purified hide collagen. It seemed of interest to compare these results with those obtained in this laboratory on the basic amino acid contents of beef achilles tendon, kangaroo tail tendon, and ichthyocol, which are types of collagen whose structures are being investigated in this laboratory. Since deamination of these proteins is one of the methods used to modify their interaction with chemicals such as metaphosphate and phosphotungstate, it seemed of immediate concern to study the effect of deamination on the specific basic amino acids. The effect on lysine has been noted by the author in the previous paper (3).

In selecting a method for the determination of arginine, the use of commercial hypochlorite, as outlined by Albanese and Frankston (4), appeared to be convenient and accurate. However, in the case of gelatin, calculation of the arginine-N as per cent of the total N from the figures given in Table I of Albanese and Frankston (4) gave a figure of 15.5%, compared with 11.82% given in Table II. This serious discrepancy prompted a thorough investigation of their method, with the

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result that it was found inapplicable to collagen hydrolyzates. A successful modification of the Sakaguchi (5) reaction is outlined in the experimental section.

Albanese, Frankston and Irby have published a convenient method for the determination of histidine by a modification of the Pauly diazo test (6). Since the solution containing the basic amino acids contains no tyrosine, their method was modified slightly by omitting the acid and permanganate treatment, thus preventing the formation of a suspension of MnO_2 which takes place on the addition of $NaNO_2$.

Experimental

The collagen-containing materials and the methods used for the determination of total nitrogen and for the separation of the basic amino acids are those described in the previous paper (3).

Determination of Arginine

The essential feature of the method is the addition of 1 ml. of hypochlorite solution of a concentration such that the maximum color develops in 30 to 75 sec. after its

TABLE I

The Arginine and Histidine Contents of Various Collagen Preparations as Determined in the Present Investigation Compared with Values Obtained from the Literature[a]

Investigator and reference	Source of collagen	Total N of moisture-free protein	Arginine content		Histidine content	
			$\frac{\text{Arginine-N}}{\text{Total N}}$	Arginine of protein	$\frac{\text{Histidine-N}}{\text{Total N}}$	Histidine of protein
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Present	Ichthyocol	17.83	17.20	9.55	1.12	0.74
Beveridge and Lucas (11)	Isinglass	18.21	16.5	9.33	1.62	1.09
Macpherson (1)	Steer hide	18.2	15.2	8.61	1.1	0.74
Present	Beef tendon	17.25	17.31	9.31	1.43	0.91
Present	Deaminized beef tendon	16.76	16.60	8.65	1.19	0.73
Present	Kangaroo tail tendon	17.45	16.70	9.02	1.32	0.85
Present	Deaminized kangaroo tail tendon	16.82	16.3	8.52	1.12	0.70

[a] The total nitrogen determinations checked within 1%, while the arginine determinations checked within 4–5%, and the histidine determinations within 3–4%. All determinations were done in duplicate.

TABLE II

A Summary of the Basic Amino Acid Contents of Various Collagen Preparations

Values are expressed as millimoles/g. of Protein[a]

Amino acid	Ichthyocol	Beef tendon		Kangaroo tail tendon		Steer hide
		Normal	Deaminized	Normal	Deaminized	
Arginine	0.546	0.533	0.497	0.518	0.490	0.494 (1)
Histidine	0.048	0.059	0.047	0.055	0.045	0.047 (1)
Lysine	0.276 (3)	0.263 (3)	0.0 (3)[b]	0.277 (3)[b]	0.0 (3)[b]	0.306 (1)
Hydroxy-lysine	0.022 (11)	0.068 (2)[c]	0.0 (2)[b]	0.068 (2)[c]	0.0 (2)[b]	0.071 (2)
Totals	0.892	0.923	0.544	0.918	0.535	0.918

[a] The figures in parentheses are references to the literature.

[b] Assuming that lysine and hydroxylysine are completely destroyed on deamination.

[c] A rough titration [according to the method outlined on p. 694 of Van Slyke *et al.* (13)] of the catholyte solutions in these two cases indicated that the amounts of hydroxylysine were roughly equal to that found by Rees (2) for hide collagen. The figure used in the above table for beef tendon and kangaroo tail tendon is that of Rees calculated for the differences in N content of the different types of collagen.

addition, and then begins to fade immediately at a rapid rate. The α-naphthol and NaOH solutions are prepared according to Albanese and Frankston (4). The hypochlorite concentrations are adjusted according to the requirements mentioned above and are usually met by using 1:5, 1:10, or 1:15 dilutions of the commercial stock hypochlorite. The directions which follow are essential for the successful use of the modified method.

X ml. of hydrolyzate and $5-X$ ml. of water are placed in a colorimeter tube followed by 1 ml. of 10% NaOH solution and 1 ml. of 0.1% α-naphthol solution. After thorough mixing, the tube is placed in a bath of cold, running tap water for 5 min. One ml. of the proper dilution of hypochlorite (determined by preliminary runs on arginine standards of 0.1–0.3 mg., and on 1–5 ml. aliquots of hydrolyzate) is added with a rapid-delivery pipette. The standards are run at the same time and the results plotted without any blank corrections. The concentration in the unknown is then obtained from the curve. The intensity of the color developed by arginine decreases linearly with the amount of arginine employed for analysis, as Jorpes and Thoren (7) had found using hypobromite as the oxidizing reagent. Therefore, at least 3 different sized aliqouts were used for each determination, and the true arginine content was obtained by extrapolation to zero hydrolyzate concentration according to the method of Brand and Kassel (8).

Results and Discussion

In Table I the results of the present investigation are compared with those obtained by other investigators on similar materials. It is appar-

ent that both types of tendon collagen have somewhat higher arginine and histidine contents than does the purified hide collagen. The arginine and histidine contents of ichthyocol are, respectively, higher and lower than those reported by Beveridge and Lucas (11). This may be a reflection of differences in starting materials and in preparation of the materials for analysis.

The protein known as collagen is that material which remains after all other known proteins have been removed by liming, tryptic digestion and successive extraction with salt solutions and water. Very little is known about the effects of these treatments on the collagen component. Therefore, it seemed of importance to determine the basic amino acid contents of tissues which yield a very high proportion of collagen in relation to the other components, prior to purification. It is likely that the figures presented in Table I are not far removed from those which will be found eventually in the purified proteins, for the following reasons: First, the precision of most colorimetric methods is of the order of 3–5%, which means that the changes in amino acid composition will have to be greater than the error in the methods to be of any significance. Secondly, the amounts of contaminating materials in tendon are very small (less than 5% of the tendon weight) and their basic amino acid contents are not unlike that of collagen (except for elastin, which is very low in the basic amino acids). Therefore, the differences seen in Table I may reflect variation in the basic amino acid content of collagen from the different species.

It is obvious, from the figures in Table I, that deamination causes a loss in arginine and histidine, as well as completely destroying lysine, as previously reported (3). The powdered beef tendon, on deamination, shows a loss of 7.1% in arginine and 20% in histidine, while the kangaroo tendon strips show corresponding losses of 5.5 and 17.5%. Steudel and Schumann (9) showed that the deamination of casein resulted in some destruction of arginine and histidine, while Wiley and Lewis (10) claimed about 50% destruction of histidine, and possibly some destruction of arginine.

For purposes of comparison, the results obtained thus far on the types of collagen reported on in this paper, and those results obtained on steer hide collagen, as reported by Macpherson (1) and Rees (2), are summarized in Table II.

It is interesting to note that, although the total basic amino acid contents of the mammalian types of collagen are almost equal, 0.92

millimoles/g., the contents of the individual basic amino acids vary somewhat. In the case of ichthyocol, the use of Beveridge and Lucas' (11) figure for histidine would bring the total for ichthyocol up to 0.91 millimoles/g.

It would seem, from the limited amount of data available,[3] that the mammal attempts primarily to fabricate a collagen of constant total basic amino acid content, and that the total can be met by some variation in the ratios of the individual basic amino acids. These results and their interpretation are in contrast with those of Block (12) for the eukeratins, where the importance in the constancy of the ratios is stressed.

The deaminized tendon collagen has a total basic amino acid content of 0.54 millimoles/g. as compared with 0.92 millimoles/g. for the intact tendon collagen.

Summary

1. The arginine and histidine contents of the following types of collagen were determined: beef tendon, deaminized beef tendon, kangaroo tail tendon, deaminized kangaroo tail tendon, and ichthyocol.

2. The arginine and histidine contents of the undeaminized tendons are somewhat higher than the values reported in the literature for purified hide collagen.

3. Ichthyocol has a higher content of arginine and a lower content of histidine than the values reported by Beveridge and Lucas.

4. Deamination of tendon collagen, besides completely destroying the terminal amino groups of lysine and hydroxylysine, causes some destruction of histidine and arginine. Deamination results in a 41% loss in total basic amino acid content.

5. From the limited data available, it would seem that the total basic amino acid content of the mammalian types of collagen does not vary significantly, although the ratios of individual basic amino acids do.

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Stereochemical Configuration and Provitamin A Activity. IX. A Comparison of All-*trans*-γ-Carotene and Pro-γ-Carotene with All-*trans*-β-Carotene in the Chick

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Introduction

Little quantitative information is available concerning the utilization of the carotenoids in the chick. Several groups of workers have indicated that the requirement for vitamin A is 8–10 times that of the rat. Sherwood and Fraps (9) found that the maintenance of white Leghorn pullets weighing 1.5 kg. is about 105 units daily or approximately 73 units/kg. Kline, Schultze and Hart (8) have reported that chicks depleted of vitamin A require a daily dose of more than 50 γ to grow to maturity while Frohring and Wyeno (6) place the minimum daily vitamin A requirement for the 8-week old Leghorn chick at 32.5 γ (65 A.D.M.A. units).

T. K. With (11) must be credited with the first extensive studies on the pro-vitamin A effect of carotenoid pigments in the chick. He has suggested that a qualitative difference obtains between the chick and the rat in the utilization of cryptoxanthin, $C_{40}H_{55}\cdot OH$, as compared with β-carotene, $C_{40}H_{56}$. Whereas cryptoxanthin has been shown to have only 58% of the pro-vitamin A activity of β-carotene in the rat (5), this investigator concluded that cryptoxanthin is actually more potent than β-carotene in the chick. Some variation in the metabolism of cryptoxanthin and carotene might be possible in the chick since among others, fowls, unlike the rat, are able to store rela-

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tively large amounts of the dihydroxy carotenes, such as lutein, and zeaxanthin, $C_{40}H_{54}(OH)_2$, in their depot fat and elsewhere. However, With did not use pure cryptoxanthin for his tests but based his conclusion on the response to the feeding of yellow corn. The chick also differs from the rat in absorbing carotenoids as such and depositing them to some extent, in the liver. This apparently may also occur with pro-γ-carotene.[4]

Recently, Johnson, Swick and Baumann (7) have failed to confirm the observation of With on the superior provitamin A activity of cryptoxanthin: on the basis of growth experiments, cryptoxanthin was shown to be even inferior to β-carotene in the chick, although it gave rise to a vitamin A storage in the liver only slightly lower than that caused by the intake of β-carotene.

The tests reported below were undertaken to study further the interrelationship of the carotenoids as provitamins A, as well as to extend our studies on the activity of stereoisomeric carotenoids to the chick (*cf.* 7). The results indicate that, as suggested first by With, the bioassay of vitamin A can be conveniently accomplished by the use of chickens. We find that the response of the chick and rat are qualitatively similar.

Methods

White Leghorn roosters obtained from the Pioneer Hatchery, Petaluma, Calif., were used. The chicks were given no food subsequent to hatching and, when received at one day of age, were immediately placed on the low-vitamin A diet. The birds were housed in equal sized groups in electrically heated brooders with raised screen floors. These brooders are maintained at 95°F. (35°C.) for the first week and then at 90°F. (32°C.) for the balance of the experiment.

Diet 1 which was used in the first series of tests is quite similar to that of With (10). It was somewhat less satisfactory than Diet 2 (used in Series II) in which $CaCO_3$ and NaCl were replaced by a salt mixture similar to that used by Johnson, Swick and Baumann (7). The composition of these diets is included in Table I.

The birds were kept on the vitamin A-low diet until they showed symptoms of A-deficiency. Although ataxia and xerophthalmia resulted in several cases, and some of the chicks died during the depletion period, the most reliable gauge of deficiency was the failure to grow. This required an average of 24 days in Series I and 21 days in Series II. At the end of this depletion period, the very light or very heavy chicks were culled and those remaining were equally distributed among the assay and controls groups. From an original consignment of 200 chicks, about 150 were available for Series II. The individual animals were provided with leg bands at the start of the tests.

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[4] To each of 8 chicks a daily dose of 0.51 mg. of pro-γ-carotene (in 0.9 ml. Wesson oil, containing 0.5% α-tocopherol) was administered for 3 consecutive days, whereupon the animals were sacrificed and the combined saponified extract of the 8 livers (85 g.) was chromatographed. As little as 0.35 mg. of pro-γ-carotene (about 3% of the amount given) was present in the chromatogram, representing the main zone.

TABLE I

Basal Diets Low in Vitamin A Used in Bioassays with Chicks

Constituent	Diet 1 *per cent*	Diet 2 *per cent*
Whole wheat (ground)	41.0	40.0
Barley (ground)	38.5	38.0
Commercial casein	10.0	12.0
Brewer's yeast (Anheuser-Busch, Strain G)	5.0	5.0
Cottonseed oil (Wesson) containing 2400 chick units of vitamin D_3/kg. diet[a]	2.0	2.0
Commercial NaCl	1.0	—
Commercial NaCl containing 0.02% KI	—	1.0
Calcium carbonate	2.4	—
Salt mixture[b]	—	2.0
Chloine chloride[c]	—	—

[a] Desynon (Winthrop), a pure crystalline preparation of vitamin D_3 was kindly furnished through the courtesy of Mr. C. Moser.

[b] Composition/100 g.: $CaCO_3$, 30 g.; KH_2PO_4, 30 g.; $CaHPO_4$, 25 g.; $MgSO_4 \cdot 7H_2O$, 9.9 g.; ferric citrate, 4.9 g.; $MnSO_4 \cdot 4H_2O$, 0.1 g.; $ZnCl_2$, 0.05 g.; $CuSO_4 \cdot 5H_2O$, 0.05 g.

[c] Choline chloride was added to Diet 2 (0.2 g./kg.).

The supplements were fed daily in 0.1 ml. of Wesson oil containing 0.5% α-tocopherol (0.5 mg. per dose). A 0.25 ml. tuberculin syringe with a blunted 18-gauge needle was used for the oral administration of the supplements. The standard β-carotene was fed at two levels, while the other carotenoids were given at three levels in Series II. In addition, there was a negative control group which received as a supplement only the Wesson oil containing 0.5% α-tocopherol daily. A positive control group was also employed concurrently, receiving excess vitamin A, *viz.*, 95 U. S. P. units daily which was present as the natural ester of vitamin A. The basal diet and water were supplied *ad lib.* The experiments were continued for 21 days. Although Series I is indecisive because too high levels of the supplement were employed, the parallelism of the growth-log dose curves with the supplements in Series II (Fig. 1) indicated that a 21-day period is satisfactory.

Results and Discussion

The results of the tests on 71 chicks used in Series I, and on 150 chicks in Series II are summarized in Table II. In Fig. 1 the growth response in Series II is plotted against the log dose of the carotenoid fed.

The average potency of pro-γ-carotene was found to be 51% of that of β-carotene. This figure is slightly higher than the values of 44% (4), and 41% (2) reported for pro-γ-carotene in the rat. All-*trans*-γ-carotene (*ex* pro-γ-carotene) had an average potency of 42% of that of β-carotene. This value is considerably higher than the figure of 28% previously

TABLE II

Summary Table Giving the Body Weights and Total Gain in Weight for Roosters Receiving Carotenoids in Cottonseed Oil Containing 0.5 mg. of α-Tocopherol Daily, or the Oil Alone (Negative Controls) or Excess Vitamin A Ester (Positive Controls)

Supplement	Dose per day γ	Average body weight in g. up to following days[a]							No. of deaths and (percentage)	Potency β-carotene =100	Av. total gain g.
		Start	3rd	7th[b]	10th	14th	18th	21st			
Series I. 9 birds/group. Average weight at start of depletion, 38 g. Depletion period, 24 days.											
All-*trans*-β-carotene	2.0	116.6	133.3	146.6	176.9	199.9(8)	247.0	279.0	1(11)		165
	4.0	112.0	128.2	148.7	176.9	211.5	256.1	284.3	0(0)		174
All-*trans*-γ-carotene	8.0	115.0	139.5	158.1(8)	187.2	219.1	264.0	297.8	1(11)		179
(*ex* pro-γ-carotene)	16.0	119.3	132.8	152.9	186.2	213.1	256.0	284.7	0(0)		165
Pro-γ-carotene	4.0	115.9	130.1(7)	145.4	162.1	186.0	219.2(6)	274.0(4)	5(56)		152
	8.0	114.2	127.1	149.9	177.7	205.4	257.8	287.1	0(0)		173
Positive controls	95[c]	114.9	144.5(8)	168.1	206.6	240.8	294.1	342.1	1(11)		223
Negative controls[d]	0.0	119.9	(6)	(5)	(3)		(2)		6(75)		

TABLE II—*Continued*

Supplement	Dose per day γ	Average body weight in g. up to following days[a]							No. of deaths and (percentage)	Potency β-carotene = 100	Av. total gain g.
		Start	3rd	7th[b]	10th	14th	18th	21st			
Series II. 15 birds/group. Average weight at start of depletion, 38 g. Depletion period 21 days.											
All-*trans*-β-carotene	1.0	144.6	—	192.3(14)	—	212.0(13)	237.3(12)	249.9(11)	4(27)		101
	2.0	143.5	—	183.8(14)	—	236.9	268.4(13)	296.0	2(14)		153
All-*trans*-γ-carotene (*ex* pro-γ-carotene)	2.5	140.6	—	184.8(11)	—	215.7(10)	238.4	243.2	5(33)	37.2	94
	3.5	143.0	—	180.0(14)	—	218.5(13)	260.5(12)	277.2	3(20)	42.5	130
	4.5	144.0	—	196.0(14)	—	236.0	278.0(13)	300.0	2(14)	45.5	155
Pro-γ-carotene	2.5	145.3	—	193.4(14)	—	237.0	253.9	272.5(13)	2(14)	55.0	124
	3.5	143.5	—	182.6	—	236.5	258.4	288.0	0(0)	51.3	144
	4.5	145.0	—	188.0	—	246.0	275.0	303.1	0(0)	47.5	158
Positive controls	95[c]	144.5	—	204.2	—	286.2	330.4	372.5	0(0)		228
Negative controls	0.0	142.6	—	(14)	—	(9)	(5)	(1)	14(93)		

[a] The figures in parentheses indicate the number of surviving birds on which the averages are made for this and subsequent periods.

[b] Weights were for 6th day in Series I.

[c] 95 U. S. P. units of natural vitamin A ester fed daily.

[d] Only 8 birds in this group.

reported with the rat for all-*trans*-γ-carotene obtained from *Mimulus* (4) and the identical result of 26% for the all-*trans*-γ-carotene prepared from the ripe berries of *Pyracantha angustifolia* (3). However, it compares excellently with the figure of 42% (2) found in the rat for the all-*trans*-isomer obtained from pro-γ-carotene by iodine catalysis (12).

Not only do the data on the gain in body weight support the fact that in the chick pro-γ-carotene is a slightly more efficient provitamin A than its all-*trans*-isomer, but also the results of survival confirm this

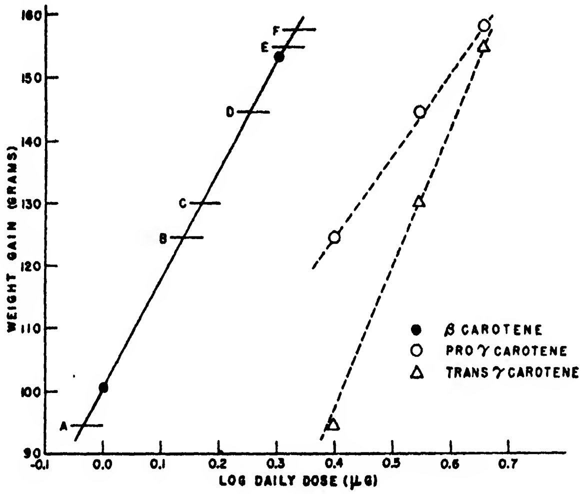

FIG. 1. Relationship of gain-in-weight to log. of daily dosage of β-carotene, all-*trans*-γ-carotene (*ex* pro-γ) and pro-γ-carotene (Series II). Points *A*, *C*, and *E* are the projections of the weight increase of the chicks receiving 2.5, 3.5, and 4.5 μg, respectively, of all-*trans*-γ-carotene daily on the β-carotene curve while points *B*, *D*, and *F* represent the projection of the weight increase of birds receiving 2.5, 3.5, and 4.5 μg, respectively, of pro-γ-carotene daily on the β-carotene curve.

statement. Whereas the fatalities were 33, 20, and 14% in the birds receiving 2.5, 3.5, and 4.5 γ, respectively of all-*trans*-γ-carotene in Series II, only 14, 0, and 0% of the chicks died during the test period when corresponding doses of pro-γ-carotene were given.

We find that the maximum requirement of β-carotene/kg body weight for a normal growth in the young chick approximate that for the young rat. The present results do not agree with some earlier reports (11) that much more vitamin A is needed in the chick (per body weight) than in the rat. In our earlier tests, it has been found that the growth

response of young rats weighing approximately 80 g. at the start of the bioassay, was 25–35 g. for the 0.5 γ daily dosage of β-carotene; and about 55 g. for the animals receiving 1.0 γ daily over a 28-day test period. In the present tests, roosters weighing 145 g. at the start of the bioassay, gained 101 g. during a 21-day period when 1.0 γ of β-carotene was administered daily. *This would indicate that, in very young animals, β-carotene is somewhat more than twice as effective for growth in the chicken as it is in the rat.*

It is realized that such direct comparisons may be open to some criticism for two reasons. First, the U. S. P. XIII diet for the rat tests differs considerably from the diet used in the present assays with chickens. Secondly, while the tests on rats were started when the animals were 45–50 days old, those on the chick commenced at an age of 20–25 days. In spite of these considerations, however, it is evident that the percentage weight increase resulting from the administration of a given amount of β-carotene is greater in the young chick than in the young rat.

Our chick tests indicate that the effective levels are considerably lower than those employed by some earlier workers (7). While our results in Series I fail to show any appreciable differences in growth between the 2.0 and 4.0 γ β-carotene levels or between the 8.0 and 16.0 γ levels of all-*trans*-γ-carotene, the growth response in Series II plotted against log dose gave a straight line when β-carotene was fed at 1.0 and 2.0 γ and all-*trans*-γ-carotene or pro-γ-carotene were given at the 2.5, 3.5, and 4.5 γ levels. These curves show a reasonable parallelism which is one of the requirements for a satisfactory assay, according to Coward (1). Moreover, another such requirement in rats seems to be fulfilled in the reported experiments with chicks, *viz.*, the basal diet and the previous period of depletion were such that 75% of the negative controls in Series I and 93% in Series II (14 of 15 birds) died before the termination of the test. In addition, the optimum growth produced by 95 I. U. of vitamin A far exceeded the figure obtained for any of the carotenoids tested. This maximum weight gain on Diet *1* averaged 223 g., and on Diet *2* it was 228 g. per chick. Johnson, Swick and Baumann (7), with a different breed of chickens, obtained 268 g. in one series and 235 g. in their second series for the optimum growth.

Finally, the most satisfactory assays would seem to be those in which the group receiving the lowest level of supplement were at a sufficiently suboptimal level so that some fatalities resulted. In the present tests,

not only is there an increased growth response at successively higher levels of intake but the survival is also progressively improved. However, it should be emphasized that the test period should be long enough so that those animals which fail to gain could be excluded from the picture because of early death. Also, a large enough number of test animals should survive to give an average gain in weight in such groups which are reliable and not influenced by abnormal variations of a few chicks. In Series II, this condition has been fulfilled by employing groups of 15 birds. With the exception of the negative control group, the minimum survival was 10 birds which gives a satisfactory mean value.

It is possible that our failure to obtain a quantitative growth response with higher doses of β-carotene in Series I may be due to the inadequacy of the basal diet. On the other hand, when Diet *2* was used, the results were more uniform and a greater growth appeared during the depletion period. This might indicate that the lower growth in Series I at the end of the depletion period (115 g. as contrasted with 145 g.) is to be ascribed to some deficiencies other than vitamin A. Further tests at several higher levels of β-carotene with the more satisfactory basal Diet *2* are now in progress.

Summary

1. The chick has been shown to be a satisfactory animal for the bioassay of provitamins A (*cf.* 10). The vitamin A requirement to produce a given percentage of growth in young animals is less for the chick than that in the young rat.

2. In the chick, pro-γ-carotene has a provitamin A potency of 51% of that of all-*trans*-β-carotene, *vs.* the corresponding figure, *viz.*, 42% for all-*trans*-γ-carotene (prepared by stereoisomerization of pro-γ-carotene). In accordance with experiments on rats reported earlier (12), this shows that the provitamin A potency of a poly-*cis* carotenoid may reach and even surpass that of the corresponding poly-*trans* compound also in the chick.

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The Effect of Insulin on the Aerobic Phosphorylation of Creatine in Tissues from Alloxan-Diabetic Rats

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It has been suggested, on indirect evidence, that the action of insulin in intermediary carbohydrate metabolism may be to establish a more efficient coupling between the processes of phosphorylation and oxidation (1). Lundsgaard (2) found, in hind limb preparations of the cat perfused with glucose-enriched blood, that insulin augmented the removal of glucose and at the same time caused a pronounced lowering of the blood inorganic phosphate. Glucose also disappeared from the blood in these hind limb preparations in the absence of insulin but the level of plasma inorganic phosphate remained unaffected. Gemmill (3,4) showed that rat diaphragm muscle equilibrated with glucose *in vitro* formed significantly greater amounts of glycogen in the presence of added insulin without an accompanying increase in the consumption of oxygen, a finding confirmed by Hechter, Levine and Soskin (5) and by Stadie (1,6). Since the entry of glucose into the metabolic cycle involves a phosphorylation by adenosinetriphosphate (ATP), Stadie suggested that the extra ATP required for the augmented glucose uptake may have arisen by some reaction catalyzed by insulin. An accelerated formation of ATP unaccompanied by a corresponding increase in oxygen consumption would imply a more efficient coupling of phosphorylation and oxidation. Studies with isotopic phosphorus (P^{32}) have disclosed that insulin injected into normal animals promotes an increased rate of phosphate uptake in the ATP and phosphocreatine fractions of muscle (7,8) and the ATP fraction of liver (9), findings consistent with the view that insulin may exert a catalytic effect on reactions concerned with the regeneration of these high energy phosphates.

It is well established that the oxidation of a number of the interme-

diates in the tricarboxylic acid cycle are coupled with the esterification of inorganic phosphate leading to the formation of high energy phosphate *via* the adenylic acid system (10–15). The results of a preliminary investigation of the relationship of insulin to phosphorylations coupled with succinate and malate oxidation are presented in this paper.

Methods

Female albino rats of a Wistar strain, ranging in weight from 200 to 250 g., were made diabetic by the subcutaneous injection of an aqueous solution of 175 mg. of alloxan monohydrate (Eastman)/kg. of body weight, following a fasting period of 48 hr. as recommended by Kass and Waisbren (16). After the injection, the animals were given food (Purina Dog Chow) and water *ad lib.* The development of diabetes was noted by the occurrence of glucosuria and polyuria. The diabetic animals receiving insulin were injected with 2 units of protamine zinc insulin subcutaneously on the day previous to the experiment and with 2 units of zinc insulin 1 hr. before sampling the tissues.

The rats were anesthetized with 1.5 cc. of 5% sodium amytal, samples of blood taken for the blood sugar determinations (performed by the Nelson modification of the Somogyi method (20)), and the animals exsanguinated by section of the aorta. The tissues to be analyzed (brain or heart muscle) were placed in liquid air and pulverized in a chilled mortar with frequent additions of the liquid air. About 500 mg. of the powdered tissue were transferred to a tared homogenizer tube, weighed, and ice-cold water added to give a final concentration of 100 mg. of tissue/ml. of the suspension. Determinations of the dry weights of 1 ml. aliquots of these suspensions gave wet to dry weight ratios ranging from 3.58 to 3.87 for brain and 3.08 to 3.25 for heart muscle. No significant differences were encountered in the ratios of wet to dry weights of brain and heart muscle in normal and diabetic animals.

The ability of these homogenates to synthesize phosphocreatine aerobically from added creatine and inorganic phosphate was tested by the assay method described by Potter (15,17,18). The components in the main compartments of the Warburg manometer vessels were added in the following order: 30 mg. of creatine hydrate (Eastman), water to make up a final volume, including the side arm constituents, of 3 ml., 0.2 ml. of 0.5 M KCl, 0.2 ml. of 0.6 M NaF, 0.1 ml. of 0.1 M phosphate (pH 7.4 with NaOH), 0.2 ml. of 1.9×10^{-4} M cytochrome c (prepared from calf heart by the method of Keilin and Hartree (21)), and 0.2 ml. of 0.012 M sodium ATP (Armour and Company, Chicago, Ill.). Two-tenths ml. of oxidizable substrate in the form of sodium succinate (0.5 M at pH 7.4) or sodium malate (0.05 M, pH 7.4) were added to this reaction mixture. Two-tenths ml. of 10% KOH were placed in the inset cup of the vessels. The tissue homogenates were placed in the side bulbs of the Warburg flasks together with 0.2 ml. of 0.1 M $MgCl_2$. This is the only particular in which the procedure differs from that outlined by Potter. Placing the tissue in the side bulb apart from the other reactants makes it less necessary to keep the vessels in ice before they are transferred to the bath, although this usual precaution was retained in the procedure. More important is the fact that, as Potter (15) found, Mg and F are "incompatible" and precipitate out when added together to the reaction mixture with the volume much less than

3 ml. No precipitation occurred when the $MgCl_2$ was tipped in from the side-arm with all constituents present in the main compartment of the vessel.

The vessels were gassed for 5 min. with 100% oxygen and equilibrated for 5 min. at 37°C., after which the contents of the side-arms were mixed in the main compartments and the oxygen consumption recorded for a period of 20 min. At the end of the incubation period, the vessels were transferred to ice, allowed to cool, and 5 ml. of ice-cold 10% trichloroacetic acid added. The contents of the vessels were filtered in the cold and aliquots of the filtrates analyzed for phosphocreatine by the method of Fiske and SubbaRow (19). No significant hydrolysis of phosphocreatine occurs on standing several hours at 0°C. in a concentration of trichloroacetic acid up to 7%. Vessels removed from the Warburg bath before mixing and after equilibration served as controls.

In each of the first series of experiments a comparative assay was made of the oxidative phosphorylation of creatine in brain or heart muscle homogenates from one normal, one alloxanized, and one insulin-treated alloxanized rat. In a second series of experiments the effect of adding an amorphous preparation of insulin (assaying 20.9 units/mg.) to brain tissue homogenates from alloxanized rats was tested. In these experiments 0.2 ml. of the insulin solution containing 18 γ (pH 3.0) was added directly to the vessels and 0.2 ml. of water acidified to the same pH, to the control vessels.

Results

The levels of phosphocreatine and ATP in a system are maintained normally by a balance between phosphorylating or energy-mobilizing reactions on the one hand, and dephosphorylating or energy-depleting reactions on the other. The addition of fluoride to the reaction medium favors the former or synthetic process, since adenosinetriphosphatase is strongly inhibited by fluoride to which the oxidative phosphorylation systems are relatively insensitive (15).

In the system investigated, the concentration of fluoride used (0.04 *M* per flask) has been shown to be more than adequate to completely block the enolase system (22,23). Since glucose is omitted from the medium, and the amount present in the homogenate which could serve as a phosphate acceptor is negligible, it may be assumed that the phosphocreatine formed arises exclusively in reactions below the pyruvate level.

The values for oxygen consumption and phosphocreatine formation in heart muscle preparations from normal, alloxan-diabetic, and insulin-treated alloxan-diabetic rats are shown in Table I. With both succinate and malate as oxidizable substrates, a marked reduction in the formation of phosphocreatine in the tissue preparations from the alloxanized rats was observed (*t* and corresponding *P* values for the significance of differences in two means are shown in the footnote of

Table I). No consistent change in oxygen consumption was found in the heart muscle preparations from these two groups of animals. The administration of insulin to alloxanized rats elevated the synthesis of phosphocreatine to values approaching and not significantly different

TABLE I

Phosphocreatine Synthesis and Oxygen Consumption in Heart Muscle Preparations from Normal, Alloxanized, and Insulin-Treated Alloxanized Rats

Rat no.	Substrate	Normal			Alloxanized			Alloxanized and treated with insulin			
		Blood sugar (mg.-%)	PC[a]	O_2[a]	Blood sugar (mg.-%)	PC	O_2	Blood sugar (mg.-%) 1[b]	Blood sugar (mg.-%) 2	PC	O_2
1	Succinate (0.033 *M*)	104	24.8	27.6	244	13.6	17.0	273	117	22.4	20.1
2		118	24.4	24.5	311	6.0	14.5	272	90	18 6	18.5
3		130	14.2	49 0	380	6.8	39.5	280	127	10.0	37.5
4		102	17.0	39.5	263	7.2	39.5	218	70	25 0	14.2
5		112	21.4	21.0	324	7.2	24.0	293	88	15.0	15.0
6		101	23.2	23.0	224	6.4	25.0	200	93	20.0	16.0
7		112	20.2	21.0	425	10.0	46.0	344	120	20.0	29.0
8		96	26.2	22.0	220	14.8	22.0	199	70	20.6	19.0
Mean ± S. E.[c]			21.42 ±1.35	28.45 ±2.93		9.00 ±1.17	28 44 ±3.89			18.96 ±1.53	21.16 ±2.18
1	Malate (0.0033 *M*)		19.5	11.0		9.8	21.3			13.0	19.6
2			18.0	10.0		2.0	16.0			10.0	46.0
3			7.2	9.0		1.0	15.5			7.0	2.0
4			11.0	12.0		3.2	6 0			10.2	3.5
5			8.8	11.5		5.0	8.0			10.0	6.0
6			12.2	9.0		4.0	10.0			11.0	5.0
7			11.2	9.5		2.2	7.5			8.8	7.5
8			14.2	9.0		9.0	14.0			12.0	8.0
Mean ± S. E.			12.76 ±1.49	10.12 ±0.39		4.53 ±1.11	12.29 ±1.93			10.25 ±0.57	12.20 ±4.82

[a] Phosphocreatine synthesis (expressed in γ of inorganic phosphate) and oxygen consumption (in μl.)/30 mg. of tissue (wet weight) during 20 min. incubation at 37°C.

[b] 1 and 2, respectively, refer to the blood sugar values before and after the injection of insulin.

[c] Standard Error of the Mean ($\sigma/\sqrt{N}$). Test for the significance of the difference between means (24); PC synthesis (normal and alloxanized) with succinate t 6.536, $P < 0.001$, with malate t 4.331, $P < 0.001$, (normal and insulin-treated alloxanized) with succinate t 1.128, $P > 0.2$, with malate t 1.521, $P > 0.1$; oxygen consumption (alloxanized and insulin-treated alloxanized) with succinate t 1.427, $P > 0.1$.

from the normal. The oxygen consumption in these heart muscle preparations was not influenced by the injected insulin.

The phosphorylation of creatine in brain tissue homogenates was found to proceed more vigorously than in the heart muscle preparations

under identical conditions. As Potter (18) found in this system, the Q_{O_2} of respiring brain tissue is also much lower than that of heart muscle, indicating the probability of a higher P/O ratio.

There appeared to be a decrease in the respiration of the brain tissue preparations from the alloxanized rates compared to the normal, although this change was less pronounced than the reduction in phosphocreatine synthesis (Table II). Brain preparations from the alloxanized animals injected with insulin showed a normal synthesis of phosphocreatine although the respiration was not significantly altered.

TABLE II

Phosphocreatine Synthesis and Oxygen Consumption in Brain Tissue Preparations from Normal, Alloxanized, and Insulin-Treated Alloxanized Rats

Rat no.	ATP[c]	Substrate	Normal			Alloxanized			Alloxanized and treated with insulin			
			Blood sugar (mg.-%)	PC[a]	O_2[a]	Blood sugar (mg.-%)	PC	O_2	Blood sugar (mg.-%) 1[b]	Blood sugar (mg.-%) 2	PC	O_2
1	0.00073 *M*	Malate	134	8 0	1.0	258	4.0	5.0	210	100	9.8	4.0
2	0.00073 *M*	(0.0033 *M*)	112	28.4	5.5	202	17.0	2.8	232	73	25.1	6.0
3	0.00073 *M*		84	24.0	7.5	324	15.2	3.5	298	90	21.9	4.5
4	0.00107 *M*		134	64.0	12.5	312	52.0	6.3	200	115	69.0	4.5
5	0.00107 *M*		104	82.0	12.0	401	58 0	6 5	251	79	73.0	13.0
6	0.00080 *M*	Succinate	95	17.0	12.3		2.2	5.3				
7	0.00080 *M*	(0.033 *M*)	104	24.3	47.5		2.4	4 8				
8	0 00087 *M*		97	27.2	9.0		21.9	14.9	—	60	29.6	12.1
9	0.00087 *M*		120	23.3	30.0		12.8	19.0	612	130	20.5	16.5
10	0.00087 *M*		90	26.2	18.3		8 8	16.5	610	105	19 0	18.0
11	0.00107 *M*		98	44.5	16.3		12.1	13.3				

[a] Phosphocreatine synthesis (expressed in γ of inorganic phosphate) and oxygen consumption (in μl.)/30 mg. of tissue (wet weight) during 20 min. incubation at 37°C.

[b] 1 and 2, respectively, refer to the blood sugar values before and after the injection of insulin.

[c] Concentration of ATP per vessel.

The importance of carefully standardizing the molarity of the ATP in these tests is demonstrated in Table II. Variations in the concentration of ATP in the reaction mixture were found to exert a marked effect on the generation of phosphocreatine.

The effect of insulin, *in vitro*, on the oxidative synthesis of phosphocreatine in brain tissue preparations from alloxan-diabetic rats is shown in Table III. An increase in the formation of phosphocreatine in the insulin-supplemented reaction mixtures was clearly demonstrated

in the presence of both malate and succinate[1] (t and corresponding P values are shown in the footnote of Table III). Insulin also stimulated respiration in the brain preparations containing succinate but did not affect the oxygen uptake in those containing malate. The increase in respiration with insulin in the presence of succinate was less pronounced than the acceleration in phosphocreatine synthesis.

TABLE III

The Effect of Insulin in vitro on the Aerobic Phosphorylation of Creatine in Brain Homogenates from Alloxan Diabetic Rats

Rat no.	Blood sugar (mg.-%)	Succinate (0.033 *M*)		Succinate and insulin (6 γ/ml.)		Succinate and malonate (0.033 *M*)		Malate (0.0033 *M*)		Malate and insulin (6 γ/ml.)		Malate and malonate (0.033 *M*)	
		PC[a]	O_2[a]	PC	O_2	PC	O_2	PC	O_2	PC	O_2	PC	O_2
1	315	14.0	9.6			2.0	0.0	17.7	10.8			7.1	7.9
2	330	11.7	16.0	19.4	20.1	0.8	0.0						
3	282	11.1	8.8	22.5	23.2			15.0	8.5	21.0	8.5	13.0	6.5
4	232	13.6	6.5	20.2	16.8			23.0	8.0	43.0	4.5		
5	220	9.4	18.9	26.0	26.5								
6	271	14.4	7.3	20.3	23.2								
7	212	9.8	27.0	16.4	32.0								
8	256							16.0	15.0	21.8	4.0		
9	312							22.0	6.3	31.0	10.0		
10	327							11.8	4.5	23.8	6.0		
11	230							11.0	8.0	22.0	6.5		
Mean ± S. E.[b]		12.00 ±1.35	13.44 ±2.68	20.80 ±1.18	23.63 ±1.96	1.40	0.00	16.64 ±1.64	8.72 ±1.18	27.10 ±1.35	6.58 ±1.03	10.05	7.20

[a] Phosphocreatine synthesis (expressed in γ of inorganic phosphate) and oxygen consumption (in μl.)/30 mg. of tissue (wet weight) during 20 min. incubation at 37°C.

[b] Standard Error of the Mean ($\sigma/\sqrt{N}$). Test for significance of insulin stimulation (pair comparison): phosphocreatine synthesis (succinate) t 5.376, $P < 0.01$, (malate) t 4.967, $P < 0.01$; oxygen consumption (succinate) t 4.799, $P < 0.01$, (malate) t 0.857, $P > 0.4$.

The addition of 0.2 ml. of 0.5 *M* sodium malonate completely suppressed both phosphorylation and oxygen consumption in brain preparations containing succinate but exerted only a partial inhibition of these processes when succinate was replaced by malate.

[1] The limit of sensitivity of insulin on this reaction has not yet been tested. It may be assumed that the activity found does not indicate the total activity of the 6 γ/ml. of insulin added, since a proportion of the insulin undoubtedly precipitates at the pH of the reaction mixture. Insulin inactivated by heat was found to be totally inactive in these tests.

Discussion

It is significant that the formation of phosphocreatine in the brain and heart muscle preparations from alloxan-diabetic rats is greatly reduced without a corresponding decrease in oxygen consumption, and that the administration of insulin to the alloxanized animals restores the ability of these tissues to synthesize phosphocreatine without causing an appreciable change in respiration. The absence of an effect of insulin on respiration was, however, more evident in the heart muscle than in the brain preparations. In the *in vitro* tests, insulin stimulated respiration in the brain homogenates in the presence of succinate but not malate. With both malate and succinate, insulin *in vitro* promoted a marked increase in the synthesis of phosphocreatine. These data indicate that, in the tissues from the alloxan-diabetic rats, the coupling between oxidation and phosphorylation is less complete than in the normal animal, and that a function of insulin may be to increase the efficiency of coupled oxidative phosphorylations.

According to our present concept of the thermodynamic role of phosphate in intermediary metabolism, the synthesis of ATP and phosphocreatine, with their energy-rich phosphate bonds, represents the principal means whereby the potential energy of carbohydrate (and presumably also of the split products of fat and protein breakdown) is transferred to energy-utilizing systems. The physiological significance of these findings is that insulin may increase the efficiency with which the potential energy of carbohydrate is conserved and made available for such endergonic processes as the synthesis of glycogen and other cellular products.

The demonstration of an *in vitro* effect of insulin on phosphorylations coupled with the oxidation of succinate and of malate demonstrates that insulin has some effect on reactions in or coupled with the tricarboxylic acid cycle but does not localize this action to a particular enzyme system. As Potter (15) has pointed out, the technique "provides a method for evaluating the ability of various tissues to oxidize different substrates with the production of phosphate bond energy, but the significance of the data cannot be interpreted as an assay of any single enzyme." The complex mechanism of phosphate uptake and transfer under aerobic conditions is little understood and the biocatalysts which serve as the transphosphorylating agents have not been identified. It is of interest that insulin was found to increase the aerobic phosphory-

lation of creatine in the presence of both succinate and malate, and that malonate completely abolishes oxygen consumption and phosphorylation only in the presence of the former. Lipmann (25) has recently discussed the possibility of the genesis of phosphate bond energy by carrier systems intermediate between the substrate and molecular oxygen. If insulin is concerned with some phosphorylation process common to both malate and succinate oxidation (and possibly other constituents in the tricarboxylic acid cycle) it is not inconceivable that its locus of action may be on some intermediate phosphate carrier system.

Acknowledgments

The authors wish to express their appreciation to Professor C. H. Best for his constant interest, counsel, and encouragement in this work. Thanks are also expressed to Dr. D. A. Scott and Dr. A. M. Fisher, of the Connaught Laboratories, for supplying samples of insulin used in these studies.

Summary

The aerobic phosphorylation of creatine during succinate or malate oxidation in heart muscle preparations from alloxan-diabetic rats was observed to be significantly less than normal. No significant difference was found in the oxygen uptake of heart muscle preparations from normal and alloxanized animals. Insulin injected into the alloxanized rats prior to tissue sampling caused no appreciable change in the oxygen consumption of heart muscle preparations but restored the ability of this tissue to synthesize phosphocreatine.

In brain homogenates from alloxan-diabetic rats there was also a marked reduction in the synthesis of phosphocreatine in the presence of succinate or malate. An apparent but not statistically significant decrease in respiration was observed in the succinate-supplemented system. The administration of insulin to alloxanized rats resulted in an accelerated synthesis of phosphocreatine but produced no consistent change in the respiration of brain.

The addition of an amorphous preparation of insulin to brain tissue homogenates from alloxan-diabetic rats was found to promote an increased synthesis of phosphocreatine during the oxidation of succinate or malate. Insulin *in vitro* also stimulated respiration in brain homogenates containing succinate but not in those containing malate.

The results indicate a direct participation of insulin in reactions in the

tricarboxylic acid cycle leading to a more efficient coupling between the processes of phosphorylation and oxidation.

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The Competitive Inhibition of Xanthine Oxidation by Xanthopterin

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Introduction

Wieland and Liebig (1) found that xanthopterin was oxidized to leucopterin in the presence of an enzyme obtained from milk or liver. This reaction is similar to the conversion of xanthine to uric acid, which is catalyzed by similar enzyme preparations. In a preliminary report (2) evidence was given that milk xanthine and xanthopterin oxidases *are* the same enzyme, since xanthopterin may act as a competitive inhibitor of xanthine oxidation. The present report is, in part, a more detailed account of these investigations. The spectrophotometric methods of Kalckar (3,4) have been extended to cover measurements of xanthine or xanthopterin oxidation at various pH values, and the relative rates of oxidation of the two substrates under optimal conditions are reported.

Materials

Xanthine (Eastman Kodak Co.). Recrystallized from water and dried in a vacuum dessicator over $CaCl_2$. Standard solution made by dissolving weighed sample in 0.05 *N* NaOH. Appropriate dilutions were made in 0.1 *M* sodium pyrophosphate buffers at various pH values.

Xanthopterin. A generous sample was furnished by the Lederle Laboratories Division of the American Cyanamid Company. Paper chromatography revealed the presence of several fluorescent spots as reported by Good and Johnson (5) in their preparations. In addition a faint spot with the Rf value of leucopterin was present. Xanthopterin concentrations are not corrected for impurities.

Xanthine Oxidase. Prepared essentially as described by Kalckar (4). Cream was separated from raw whole milk at + 5°C. The whey used as the starting material was obtained by removal of the casein clot following treatment of the skim milk with a commercial rennin preparation.

Methods

Spectrophotometric Measurements

A Beckman spectrophotometer (Model DU) was used. Wave lengths were calibrated using a mercury arc. Density readings were checked with standard phthalate as described by Ewing and Parsons (6). All measurements were made in 1 cm. cells.

Determination of Oxidase Activity with Xanthine as the Substrate

The increase in density at 290 mμ as described by Kalckar (3) was used to follow the conversion of xanthine to uric acid. The degree of density change (ΔE) per unit of xanthine converted was found to vary markedly with pH, a fact not emphasized by Kalckar. This variation is due largely to the increase in absorption of xanthine at 290 mμ with increasing pH in the range from 6.5 to 9.0. The density changes found are shown in Table I.[1] These values were used in calculating rates of xanthine oxidation.

TABLE I

Density Changes with the Enzymatic Conversion of Xanthine to Uric Acid at Various pH Values

Reactions were run in 0.1 M sodium pyrophosphate buffers. Corrections were made for enzyme and buffer absorption.

pH	Density change $\Delta E_{290}/\gamma$ xanthine/ml.
6.4	0.073
7.0	0.069
7.4	0.063
7.9	0.055
8.4	0.049
8.8	0.047

The presence of xanthopterin does not interfere with the method of following xanthine oxidation, since the xanthopterin to leucopterin conversion is not accompanied by absorption changes at 290 mμ.

Determination of Oxidase Activity with Xanthopterin as the Substrate

The change in density at 330 mμ can be used to follow xanthopterin oxidation. In this case only slight variation of ΔE with pH was noted. The values found for $\Delta E/\gamma$ xanthopterin/ml. converted to leucopterin are: 0.027 at pH 6.4, 0.030 at pH 7.4, and 0.033 at pH 8.4.

[1] The ΔE values are calculated from xanthine concentrations based on a weighed sample as described. If xanthine concentrations were determined by absorption at 270 mμ, using the molar extinction coefficient obtained from the curves reported by Stimson and Reuter (7), then the ΔE values of Table I would be multiplied by a factor of 1.15. The molar extinction coefficient of xanthine at pH 7.0 found in the present investigation was 9,600 at 270 mμ, instead of 11,000 as reported by the above authors.

Results

The oxidation of xanthine in the presence of the milk enzyme was found to proceed much more rapidly than the oxidation of xanthopterin. To compare the relative rates under optimal conditions for each substrate, the pH optima were determined (Fig. 1). It can be seen that, with xanthine as the substrate, there is a fairly sharp optimum near pH 8.5, whereas, with xanthopterin, there is little change in activity over the broad range from pH 6.5–8.0. The maximal rate for xanthine oxidation is about 25 times that for xanthopterin oxidation, since the enzyme concentration in curve B is 10 times greater than in curve A.

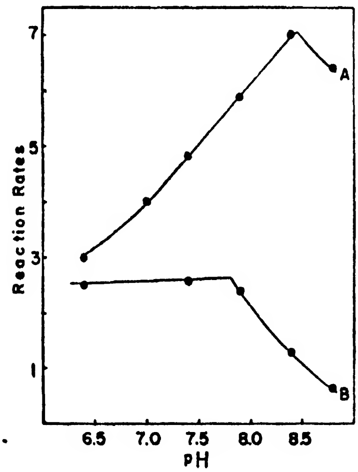

Fig. 1. The pH optima for milk xanthine oxidase with xanthine or xanthopterin as substrate. Curve A. Substrate xanthine at concentration of 5.4×10^{-5} *M* in 0.1 *M* sodium pyrophosphate buffers. Reaction rates given in terms of $\mu M \times 10^{-3}$/ml. oxidized/min. Rates calculated from density change at 290 mμ/min. Curve B. Substrate xanthopterin at concentration of 2.6×10^{-5} *M* in same buffer. Reaction rates as above. Rates calculated from density change at 330 mμ. Enzyme concentration 10 times that in A.

This result differs from the findings of Wieland and Liebig (1), who found the rate only 5 times greater with xanthine; however, their enzyme source presumably was liver, and this may account for the difference.

If the same enzyme were involved in the oxidation of xanthine and xanthopterin, it should be possible to demonstrate competitive phenom-

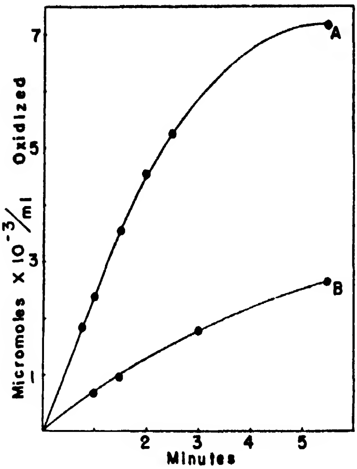

FIG. 2. The inhibition of xanthine oxidation by xanthopterin. Reaction mixture in A: 7.2×10^{-6} M xanthine in 0.1 M sodium pyrophosphate buffer at pH 7.9 plus enzyme. Reaction mixture in B the same plus xanthopterin at a concentration of 6.2×10^{-6} M. Rates of xanthine oxidation calculated from density changes at 290 mμ in each case.

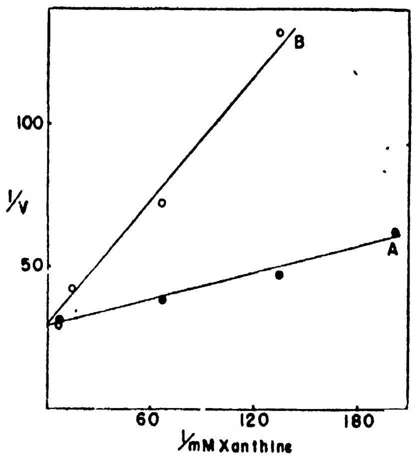

FIG. 3. Determination of the type of inhibition of xanthine oxidation by xanthopterin. In curve A the reciprocals of xanthine concentration are plotted against the reciprocals of initial velocities ($1/\Delta E_{290}$/min.). Curve B is the same type of plot for xanthine oxidation rates in the presence of xanthopterin at a concentration of 6.2×10^{-6} M. All measurements at pH 7.9.

ena when reaction mixtures contain both substrates. Xanthopterin was found to be an effective inhibitor of xanthine oxidation (Fig. 2), and the inhibition was of the competitive type (Fig. 3). Using the method of Lineweaver and Burk (8) the dissociation constant[3] of xanthine with the enzyme was estimated to be 5.3×10^{-6}. With this value of K_s, the dissociation constant of the enzyme-inhibitor complex (K_i), was 1.6×10^{-6}. Xanthopterin-7-carboxylic acid inhibited xanthine oxidation to about the same degree as xanthopterin at the same concentrations.

Discussion

Kalckar reported inhibition of xanthine oxidase by pteroylglutamic acid (4) but found later that the inhibition was due to the presence of the photofission product, 2-amino-4-hydroxy-6-pteridylaldehyde (9). The latter compound is an extremely active inhibitor of this enzyme (10). It is unlikely that xanthopterin would contain any of the 6-pteridylaldehyde. The inhibition of xanthine oxidation by xanthopterin is presumably due to xanthopterin itself, a substrate having a lower dissociation constant with the enzyme than that of xanthine.

Summary

1. Density changes found for the enzymatic conversion of xanthine to uric acid at various pH values are reported.

2. The pH optimum for xanthine oxidase with xanthine as a substrate is near pH 8.5. With xanthopterin as a substrate there is a broad optimum between pH 6.5–8.0.

3. The relative rates of xanthine and xanthopterin oxidation in the presence of the milk oxidase are approximately 25:1.

4. Xanthopterin has a lower dissociation constant with respect to the enzyme than does xanthine and acts as a competitive inhibitor of xanthine oxidation.

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[3] At the low concentrations of xanthine that must be used in a study of this type, determinations of initial reaction-velocities are subject to error. The constant of 7.0×10^{-6} given in the preliminary report (2) has been revised after reinvestigation.

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Electrophoretic and Serological Properties of the Non-Dialyzable Growth Products of *Vibrio cholerae* [1]

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Introduction

The development of a culture method capable of producing massive growth of *V. cholerae* in completely dialyzable media (1,2,3,4) has made possible the easy isolation of large quantities of the non-dialyzable products of growth, richer in immunizing antigens than those of cultures prepared by the older methods (5). These products, consisting largely of proteins and polysaccharides, make the bacterial filtrate an effective prophylactic (4). The polysaccharides, which are devoid of antigenic activity in the rabbit (6,7), can be isolated from the bacterial filtrates by elimination of the proteins (8).

By means of the Tiselius electrophoresis apparatus (9), an investigation has been made of the intact growth products from several strains of the cholera vibrio, and of the polysaccharides separated from those products. The electrophoretic fractions were tested serologically to determine the constituents responsible for the antigenic and toxic activity.

Experimental

Eight authentic cholera strains (10), 6 of the Inaba (original) type and 2 of the Ogawa (variant) type (11), were examined. The organisms were grown in 15-l. lots of a completely dialyzable casein digest medium containing glucose and inorganic salts (1). The culture was killed by the addition of phenyl mercuric nitrate, and the bacterial debris was removed in a Sharples supercentrifuge. The volume was reduced to 300 ml. by boiling at 30°C. or less *in vacuo*, and the constituents of the medium were removed by dialysis. The dialysis bags were then suspended in a current of warm air to concentrate the solution to a final volume of 100 ml.

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[1] Most of the work reported in this publication was done in 1943 and 1944.

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The concentrated bacterial filtrate contained the following ingredients: the non-dialyzable extra-cellular products of metabolism greatly concentrated, possibly traces of constituents of the medium, and intracellular matter released by autolysis before centrifugation. This last was stringently limited by harvesting almost immediately following cessation of logarithmic growth. Since the culture method is remarkably efficient, the actual incubation period, and consequent opportunity for autolysis, was reduced to a minimum. It was extended, of course, by the period of contact with the sterilizing agent; and, as indicated elsewhere in this paper, there is some reason to suppose that a significant amount of intracellular matter may have found its way into solution before centrifugation, especially in hot weather.

For separation of the polysaccharide (8), the concentrated bacterial filtrate was acidified with acetic acid to a final concentration of $N/20$, and boiled to precipitate the protein; the filtrate was then dialyzed acid-free.

All solutions were dialyzed against several changes of buffer solution of pH 6.9 containing 0.15 M NaCl and 0.02 M sodium phosphates. Following electrophoresis at 12 ma. to maximum separation of the boundaries, the contents of the sections of the U-tube were removed for further study. The composition of the solutions with respect to the electrophoretic constituents was computed from the area of the electrophoretic diagrams, assuming the value 0.00180 for the specific refractive increment of all constituents. The mobilities were calculated from the positions of the ordinates bisecting the curve areas (12).

Results of Electrophoresis Experiments

Growth Products of V. cholerae

Representative results obtained by electrophoresis of the growth products of *V. cholerae* are given in Figs. 1 and 2 and in Table I. The growth products from strains 44 and 46 of the Inaba type and strain 49 of the Ogawa type gave clear-cut, consistent patterns containing 3 main boundaries, A, X, and B, each of which separated into two parts. B-1, the rapidly migrating portion of B, was the most prominent boundary in the pattern. X-2, the slower, very sharp portion of X, was associated with the opalescence of the solution.

All other preparations of the growth products contained the same 3 constituents, but the boundaries, particularly of the X constituent, were less sharply defined (see strain 35, Fig. 1). The growth products of the same strain prepared at different times showed greater variation than those of different strains prepared at the same time. The loss of definition, particularly of the X boundary, could be correlated with hot weather and consequent possible enzyme action during processing of the bacterial filtrate. No evidence was found for specific strain differences. The similarity of the growth products from vibrios of the

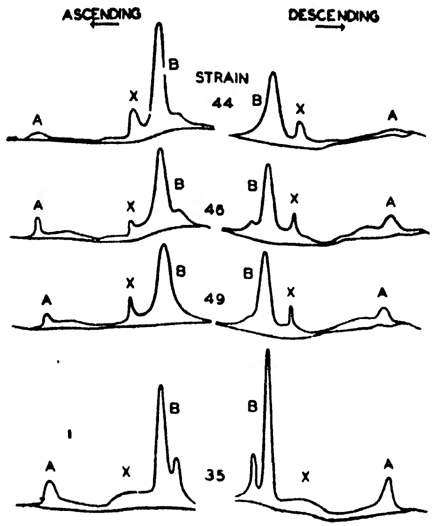

FIG. 1. Electrophoresis diagrams of the intact growth products of strains 44, 46, 49, and 35 of *V. cholerae*. Electrophoresis for 3 hr. at 12 ma. Tracings of Longsworth diagrams. Strains 44, 46, and 35 are of the Inaba (original) type, strain 49 of the Ogawa (variant) type.

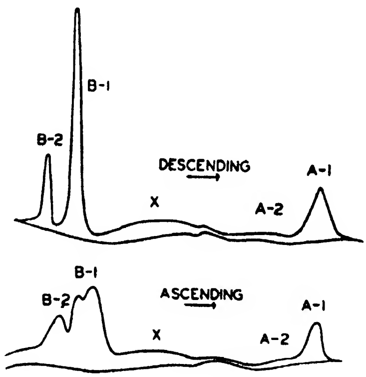

FIG. 2. Electrophoresis diagrams of the intact growth products of strain 35 of *V. cholerae*. This is the same preparation as 35 of Fig. 1, but at twice the concentration. Electrophoresis for 13.5 hr. at 12 ma. Tracings of Longsworth diagrams.

Inaba and Ogawa types (11) is significant in view of the close relationship between the types, and the ease with which *V. cholerae* produces variants under both natural and artificial conditions (10).

After prolonged electrophoresis at higher concentrations, the rising

B-1 boundary separated into two parts (Fig. 2, Table I). This phenomenon may indicate complexity of the B-1 constituent and interaction of the sub-constituents either with one another or with some other component of the mixture.

TABLE I

Electrophoretic Analysis of the Growth Products of V. cholerae

Strain		Electrophoretic constituent						
		A-1	A-2	X-1	X-2	B-1	B-2	Total
49	Mobility, descending arm	12.5	10.2	—	4.1	1.8	0.7	
*(41-C2)	Mobility, ascending arm	12.3	9.5	—	4.9	2.0	0.8	
Ogawa	Refractive increment ×10⁵	5.2	5.6	—	7.4	26.9	6.0	51.5
	Concentration in mg./ml.	0.29	0.31	—	0.41	1.50	0.33	2.88
	Percentage composition	10	11	—	14	53	12	100
46	Mobility, descending arm	12.8	10.5	5.8	4.8	2.3	0.6	
*(35-C5)	Mobility, ascending arm	13.0	10.6	6.3	5.4	2.5	1.0	
Inaba	Refractive increment ×10⁵	5.0	5.0	2.9	4.8	24.4	5.2	47.3
	Concentration in mg./ml.	0.28	0.28	0.16	0.27	1.36	0.29	2.64
	Percentage composition	11	11	6	10	51	11	100
44	Mobility, descending arm	13.0	—	6.6	4.2	2.0	0.8	
*(35-C3)	Mobility, ascending arm	13.1	—	6.0	4.4	2.2	0.7	
Inaba	Refractive increment ×10⁵	5.4	—	4.1	6.6	30.0	5.2	51.4
	Concentration in mg./ml.	0.30	—	0.23	0.37	1.67	0.29	2.85
	Percentage composition	11	—	8	13	58	10	100
44-3	Mobility, descending arm	14.4	11.0	6.9		2.8	1.8	
*(35-C3)	Mobility, ascending arm	14.7	10.9	5.4		2.7	0.7	
Inaba	Refractive increment ×10⁵	3.3	2.5	3.9		7.4	2.9	20.0
	Concentration in mg./ml.	0.18	0.14	0.22		0.41	0.16	1.11
	Percentage composition	16	13	19		37	15	100
42	Mobility, descending arm	12.8	10.6	—	3.9	2.6	1.4	
*(35-C1)	Mobility, ascending arm	12.8	10.1	—	3.5	2.6	1.4	
Inaba	Refractive increment ×10⁵	13.2	14.9	3.1	4.5	5.4	12.0	53.1
	Concentration in mg./ml.	0.73	0.83	0.17	0.25	0.30	0.67	2.95
	Percentage composition	25	28	6	8	10	23	100
35	Mobility, descending arm	13.2	11.1	5.4		2.0	0.7	
*(35)	Mobility, ascending arm	13.1	11.2	5.4		2.4	1.1	
Inaba	Refractive increment ×10⁵	9.3	5.0	15.5		29.2	11.0	70.0
	Concentration in mg./ml.	0.52	0.28	0.86		1.62	0.61	3.89
	Percentage composition	13	7	22		42	16	100
35	Mobility, descending arm	12.6	10.7	5.2		2.2	1.0	
*(35)	Mobility, ascending arm	13.1	11.0	6.0		3.5, 2.6	1.7	
Inaba	Refractive increment ×10⁵	20.3	9.1	28.5		18.0, 36.0	25.2	137.1
(2→1)	Concentration in mg./ml.	1.13	0.51	1.58		1.0, 2.0	1.4	7.62
	Percentage composition	15	7	21		13, 26	18	100

* Strain designation of the National Institute of Health.

Mobilities, computed from the positions of the ordinates bisecting the curve areas, are given in units of 10^{-5} cm./sec. volt/cm.

In computing the cencentrations in mg./ml. and the percentage composition, the value 0.0018 was assumed for the specific refractive increment of all constituents.

At the pH, 6.9, of these experiments, 0.3% formaldehyde, which converts toxins to toxoids by transformation of amino groups to methylol amino groups, caused no change in the elctrophoretic pattern.

Polysaccharides of V. cholerae

The electrophoretic patterns showed clearly the relationship of the polysaccharides to the growth products from which they had been separated. The X constituents, however, were missing, and the A and B constituents had consistently higher mobilities than in the intact growth products (Table II). The B-1 boundary was less prominent; in general the A constituents predominated instead. On prolonged electrophoresis the A-1 boundary separated into two parts in the rising

TABLE II

Electrophoretic Analysis of Polysaccharides from V. cholerae

Strain		Electrophoretic constituent							
		A-1a	A-1b	A-2	A-3	B-1	B-2a	B-2b	Total
35	Mobility, descending arm	13.9		11.7	—	—	2.2	1.9	
[a](35)	Mobility, ascending arm	14.4	12.9	11.8	—	—	2.3		
Inaba	Refractive increment $\times 10^5$	9.7	6.6	18.8	—	—	14.9	14.9	64.9
	Concentration in mg./ml.	0.54	0.37	1.03	—	—	0.83	0.83	3.60
	Percentage composition	15	10	29	—	—	23	23	100
28	Mobility, descending arm	13.5		11.3	—	—	1.7	1.2	
[a](41-A6)	Mobility, ascending arm	14.2	13.0	11.3	—	—	2.4		
Ogawa	Refractive increment $\times 10^5$	4.5	7.5	32.4	—	—	20.0	12.0	76.4
	Concentration in mg./ml.	0.25	0.42	1.80	—	—	1.11	0.67	4.25
	Percentage composition	6	10	42	—	—	26	16	100
20	Mobility, descending arm	13.9		—	—	3.5	1.9		
[a](35-A5)	Mobility, ascending arm	13.5		—	—	3.6	1.9		
Inaba	Refractive increment $\times 10^5$	8.7		—	—	4.5	6.2		19.4
	Concentration in mg./ml.	0.49		—	—	0.25	0.34		1.08
	Percentage composition	45		—	—	23	32		100
3	Mobility, descending arm	13.6		12.8	—	—	2.8		
	Mobility, ascending arm	12.7		12.3	—	—	3.0		
Inaba	Refractive increment $\times 10^5$	23.6		20.0	—	—	7.8		51.4
	Concentration in mg./ml.	1.31		1.11	—	—	0.43		2.85
	Percentage composition	46		39	—	—	15		100
3A	Mobility, descending arm	13.4		—	—	3.3	2.0		
	Mobility, ascending arm	12.6		—	—	3.6	2.1		
Inaba	Refractive increment $\times 10^5$	20.3		—	—	9.1	7.4		36.8
	Concentration in mg./ml.	1.13		—	—	0.51	0.41		2.05
	Percentage composition	55		—	—	25	20		100
3B	Mobility, descending arm	13.5		13.1	9.1	—	1.8		
	Mobility, ascending arm	13.6		12.1	9.9	—	2.0		
Inaba	Refractive increment $\times 10^5$	8.9		6.2	3.9	—	5.6		24.6
	Concentration in mg./ml.	0.49		0.34	0.22	—	0.31		1.36
	Percentage composition	36		25	16	—	23		100

[a] Strain designation of the National Institute of Health.

Mobilities, computed from the positions of the ordinates bisecting the curve areas, are given in units of 10^{-5} cm./sec. volt/cm.

In computing the concentrations in mg./ml. and the percentage composition, the value 0.0018 was assumed for the specific refractive increment of all constituents.

arm while the B-2 boundary separated into two parts in the descending arm. These anomalies may indicate complexity and interaction of the type postulated above for the B-1 constituent of the intact growth products.

The polysaccharides most nearly similar in composition were obtained from strain 35 of the Inaba type and strain 28 of the Ogawa type (Table II). This is further indication that the immunological differences between the two types are not reflected in the gross electrophoretic behavior of the products of growth.

From comparison of the patterns, it appears that, in the intact growth products, the X constituents may have consisted almost entirely of protein (or of other substances, such as lipides, which might also be removed by boiling and filtration), while the A constituents probably contained a preponderance of polysaccharide; the B constituents may have contained more nearly equivalent proportions of each. The protein associated with the A and B constituents was apparently combined with polysaccharide in such manner as to diminish the mobility of the latter. From the electrophoretic analyses alone, antigenic activity might be expected of the X constituents, since they, like the antigenic activity, were found in the bacterial filtrates but not in the polysaccharides, and possibly of the B-1 constituent in view of its apparently high protein content.

The Antigenicity and Serological Activity of the Growth Products of V. cholerae

Serological tests were restricted to cholera strains of the Inaba (original) type. For determination of the antigenicity of the various constituents, the electrophoretically separated fractions of the growth products of strain 35, the composition of which is given in Table III, were injected intravenously into normal rabbits. Six doses of 1 ml. each were injected at intervals of 3 or 4 days; the blood was drawn a week after the final injection.

The animals receiving Fraction 2 (the unfractionated growth products) and Fraction 3 (lower cathode section) developed areas of severe irritation near the injection site, while those receiving Fractions 1 and 4 (upper anode and upper cathode sections, respectively) did not. The substance responsible for the skin irritation must consequently be associated with the X constituent (Table III).

The sera obtained from these rabbits were tested for precipitin activity against the unfractionated growth products, the A constituent, the B constituent, and a mixture of the A and X constituents; typhoid immune serum was used as a control. The sera were also tested against the electrophoretic fractions of the polysaccharide. Ring tests were made first; then the tubes were shaken and stored for estimation of the precipitates. The results were negative for the controls and for all the polysaccharide fractions. The results of the other tests are shown in Table III.

TABLE III

Serological Activity of Electrophoretic Fractions of Growth Products. V. cholerae, Strain 35

Antigens used for preparation of rabbit antisera						Inflammation at injection site	Precipitin reactions of the resulting antisera with the antigens listed below			
Immunizing fraction	Composition						Unfractionated	A_1+A_2	B_1+B_2	A_1+A_2+X
	A_1	A_2	X	B_1	B_2					
1. Upper anode $\Delta n \times 10^6$ mg./ml.	9 0.50	4 0.22	0 0	0 0	0 0	–	±	±	±	±
2. Unfractionated $\Delta n \times 10^6$ mg./ml.	14 0.78	7 0.39	21 1.17	39 2.17	12 0.67	++	++	–	+++	++
3. Lower cathode $\Delta n \times 10^6$ mg./ml.	0 0	1 0.06	20 1.11	38 2.11	12 0.67	++++	++++	±	+	+
4. Upper cathode $\Delta n \times 10^6$ mg./ml.	0 0	0 0	3 0.17	30 1.67	12 0.67	–	++++	–	+++	++

It appears that, in the intact growth products of strain 35, the A constituents had no power either to elicit antibody formation in the rabbit or to react to antiserum against the unfractionated growth products, and may, therefore, be considered non-antigenic. The B constituents elicited antibodies *in vivo,* as is shown by the fact that antisera against fractions 2, 3, and 4, which contained both the X and B constituents, reacted with the B constituents alone. The antigen A + X, which may be considered pure X from the immunological viewpoint, since A is non-antigenic, reacted with the 3 antisera against the X and B constituents about as well as did the B constituents. The unfractionated growth products, which contained both the X and B constituents, gave,

in general, a stronger reaction with these antisera than either constituent alone.

Similar results were obtained when the fractions of the growth products and the polysaccharide of strain 35 were tested for precipitin activity against the serum of a rabbit immunized against the intact vibrios of strain 35, using normal rabbit serum as a control. Further experiments with the fractions of the growth products of strain 46 against the antisera to the fractions of strain 35 showed that strain 46 produces antigenic factors which are immunologically the same as those in strain 35. It appears, therefore, that, in the intact growth products of *V. cholerae*, at least from strains of the Inaba type, both the X and B constituents are antigenic.

TABLE IV

Effect of Heat on the Serological Behavior of the Growth Products of Strain 35

Fraction	Approximate concentration of each constituent relative to unfractionated growth products				Reaction with O antiserum (O) and with serum against whole organisms (W)									
	A_1	A_2	X	B	Unheated		Unheated		30 min. at 58 C.		2 hr. at 100 C.		2 hr. at 100 C.	
					O	W	O	W	O	W	O	W	O	W
Unfractionated	100%	100%	100%	100%	+	++	+	+	+	++	++	++	++	+
Upper anode	95%	50%	—	—	−	−	−	−	−	−			−	−
Lower anode	100%	100%	60%	5%		+		+		++		−		−
Bottom section	100%	100%	100%	100%	++	+	++	++		++				
Lower cathode	5%	50%	100%	100%		+		++		++		+		+
Upper cathode	−	−	40%	95%	++	++	++	++	++	++			++	++

O antiserum diluted 1/20; serum against whole organisms diluted 1/5.

To differentiate between the heat-labile H antigen, which is common to both the cholerigenic vibrios and a large number of non-cholera vibrios, and the heat-stable O-group 1 antigen, which has a much narrower specificity (10), portions of the growth products of strain 35 were subjected to heat treatment. The electrophoretic fractions obtained from them were tested for precipitin activity against rabbit antisera to whole vibrios and to the O antigen; typhoid immune serum was used as a control. Heating at 58°C. for 30 min., the treatment sufficient to destroy the capacity of the vibrios to agglutinate with H antibody, produced no change in either the electrophoretic pattern or the serological behavior (Table IV). Heating at 100°C. for 120 min., the

treatment required to destroy the ability of the vibrios to elicit H antibodies (13), caused partial fusion of the A-1 and A-2 boundaries, and destroyed the serological activity of the lower anode fraction (Table IV). This fraction from an immunological point of view may be considered practically pure X constituent. The heat-labile antigens, including the H antigen, thus appear to be associated with the X constituents, and the heat-stable O-group 1 antigen with the B constituents.

Most of the symptoms of cholera (14) are caused by a thermolabile toxin which is dialyzable through collodion and cellophane membranes (15,16,17). It is liberated from the vibrios and from the growth products by either spontaneous autolysis (17) or chemical treatment (16). Much of it may have been lost from the preparations here described because of the prolonged dialysis. In the freshly liberated growth products, however, it is probably associated with the X constituent, for the X constituent gave evidence of both toxicity and sensitiveness to autolytic change.

Summary

The intact growth products from several strains of *V. cholerae* were shown by electrophoresis to contain three main constituents, A, X, and B, each of which usually separated into two or more sub-constituents. The polysaccharides isolated from the growth products contained the A and B constituents also, but in different proportions; the X constituents were missing. In both the intact growth products and the polysaccharides there was evidence for reversibly dissociable interaction of the sub-constituents.

The X and B constituents of the intact growth products possessed antigenic activity. The X constituent produced inflammation at the site of injection. The heat-labile antigens appeared to be associated with the X constituents, the heat-stable O-group 1 antigen with the B constituents.

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The Formation of Glucose Diphosphate by *Escherichia coli*

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Introduction

Glucose-1,6-diphosphate[1] has been found to act as a coenzyme in the reaction:

$$\text{Glucose-1-phosphate} \rightleftharpoons \text{glucose-6-phosphate}$$

when it is catalyzed by yeast or animal tissue phosphoglucomutase (2). During this latter investigation it was observed that glucose-1-phosphate solutions became contaminated with appreciable amounts of glucose diphosphate when stored for some time in the cold. From such solutions it was possible to isolate several microorganisms. Of these, *Escherichia coli* was found to be responsible for the formation of glucose diphosphate. Several strains of this organism were examined, as well as *Aerobacter aerogenes* and *Klebsiella pneumoniae*. The synthesis of glucose diphosphate occurred in the presence of any of these, whereas no synthesis was obtained in preliminary tests with *Bacillus cereus*, *Bacillus alkaligenes*, *Sarcina conjunctivae*, *Serratia marcescens* and *Staphylococcus aureus*.

This paper represents the results of a study of the formation of glucose diphosphate and the fermentation of phosphoric esters by living *E. coli*. Extracts of *E. coli*, which catalyzed the synthesis of glucose diphosphate, were prepared and purification of the active system was attempted. The mechanism of the reaction and the action of factors which influenced its rate were also studied.

Methods

Cultures

For small scale experiments, *E. coli* was cultivated on agar slants. After 24 hr. incubation, the bacterial growth was washed off, centrifuged and resuspended in

[1] The structure of this substance has been confirmed by synthesis from silver phosphate and 1-bromo-2,3,4-triacetyl-6-diphenylphosphonoglucose by Repetto *et al.* (1).

water to a density corresponding to an extinction coefficient of E. = 0.8 for 1 cm. at 470 mμ. For larger scale experiments, the bacteria were harvested from peptone broth cultures after 20 hr. of incubation at 30°C. with aeration. The bacterial mass was separated by means of a continuous centrifuge and then stored in the frozen state until used. Extracts obtained after some days of storage were often more active than those obtained from fresh bacteria.

Substrates

Glucose-1-phosphate was prepared as described by Sumner and Somers (3), glucose-6-phosphate according to Colowick and Sutherland (4), and fructose diphosphate after Neuberg and Lustig (5). The preparation of glucose diphosphate has been previously described (2).

Analytical Methods

Glucose diphosphate was estimated by determination of its coenzymatic activity with a yeast phosphoglucomutase (2). Inorganic phosphate was determined by the procedure of Fiske and SubbaRow (6). The inorganic phosphate liberated during 7 min. of hydrolysis at 100°C. by 1 *N* acid is referred to as acid-labile phosphate, and that portion of the organic phosphate not hydrolyzed under similar conditions is referred to as acid-stable phosphate. Glucose was determined by the Somogyi (7) procedure using the Nelson reagent (8). Fructose was determined according to the directions of Roe (9).

Results

The Formation of Glucose Diphosphate by Living E. coli

When the bacteria were suspended in a glucose-1-phosphate solution, an accumulation of glucose diphosphate occurred which reached a maximum and then decreased. The time at which the maximum was obtained was variable and was found to depend on the amount of bacteria added and on the initial concentration of the substrate. A graphic representation of a typical experiment appears in Fig. 1.

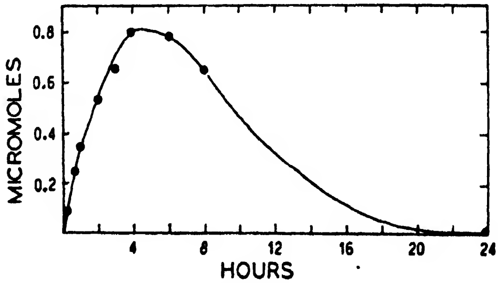

Fig. 1. Glucose diphosphate formation by living *E. coli*. Incubation at 37°C. of 20 μ*M* of glucose-1-phosphate, 1 ml. of *M*/15 phosphate buffer of pH 6.5 and 0.25 ml. of a suspension of *E. coli*. Total volume, 3.25 ml. Results in μ*M*.

The formation of the diphosphate under aerobic and anaerobic conditions was approximately the same. The maximum amount of diphosphate formed corresponded to the conversion of about 4% of the initial amount of monophosphate added.

The formation of glucose diphosphate was measured with substrates other than glucose-1-phosphate. No formation was detected from glucose (with or without added inorganic phosphate), glucose-6-phosphate, fructose diphosphate, saccharose, lactose, or maltose. The culture liquid remaining after centrifuging off the bacteria did not catalyze the formation of glucose diphosphate from the monophosphate.

The Fermentation of Phosphoric Esters

Manometric experiments showed that glucose-1-phosphate, glucose + inorganic phosphate, and glucose were rapidly fermented by *E. coli* (Table I) and also by *Aerobacter aerogenes* and *Klebsiella pneumoniae.*

TABLE I

Acid Formation by Living E. coli

CO_2 evolution measured in Warburg manometers containing: 0.025 *M* $NaHCO_3$, 4 μM of substrate and 0.4 ml. of a suspension of *E. coli*. Total volume: 2 ml. Temperature: 37°C. Results in μl. Gas: Nitrogen with 5% CO_2.

Substrate	25 min.	50 min
None	0	0
Glucose	30	60
Glucose+phosphate	33	67
Glucose-1-phosphate	37	75
Glucose-6-phosphate	17	35
Fructose diphosphate	7	8
Glucose diphosphate	8	10

Usually, glucose-1-phosphate was utilized at a rate slightly faster than the others. The rate of fermentation of glucose-6-phosphate was lower, while that of fructose diphosphate and glucose diphosphate was nearly undetectable.

These organisms differ from yeast, where glucose-1-phosphate is not fermented by intact cells. Evidently, there is a difference in permeability, since in *E. coli* glucose-1-phosphate is not hydrolyzed before entering the cells, for, if such were the case, glucose plus inorganic phsophate should be equivalent to glucose-1-phosphate in all respects. However, only the latter gives rise to glucose diphosphate.

Glucose Diphosphate Formation in Cell-Free Extracts

To study the mechanism of formation of glucose diphosphate from glucose-1-phosphate, it was deemed necessary to separate the enzyme system involved in this reaction. Crude extracts prepared from *E. coli*, acting on glucose-1-phosphate, were found to produce glucose diphosphate, together with considerable amounts of inorganic phosphate and reducing substances.

If the reaction is formulated as:

$$2 \text{ glucose-1-phosphate} \rightarrow \text{glucose-1,6-diphosphate} + \text{glucose},$$

the molar ratio: glucose diphosphate/glucose should be 1. Actually, with the crude extracts, this ratio was found to be 0.01. The amount of inorganic phosphate liberated was roughly the same as that of reducing substances calculated as glucose, evidence which suggested that the main

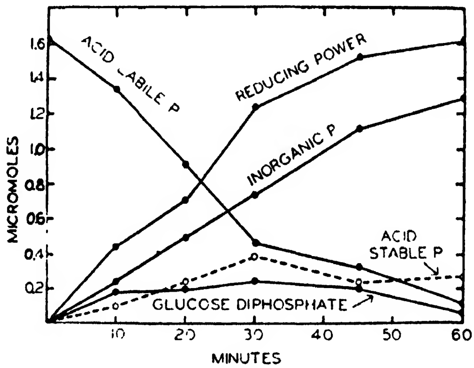

FIG. 2. Chemical changes produced by the enzyme. 0.25 ml. samples containing 1.7 μM glucose-1-phosphate, 2 μM cysteine and 0.03 ml. of purified enzyme. Temperature, 37°C. Reducing power referred to a glucose standard.

contaminating enzyme was a phosphatase. The increase in value of this ratio was, therefore, utilized as an index of the degree of purification of the diphosphate-forming enzyme. As can be observed in Fig. 2, the value of this ratio, with the same enzyme preparation, was found to depend on the length of incubation, and was approximately constant only during the first few minutes of the reaction.

Preparation of Cell-Free Extracts

Several procedures were attempted: Cytolysis with toluene, grinding with glass powder (10), and extraction of acetone-dried cells. The latter procedure was adopted,

since it was found to yield more reproducible results. The dried bacteria, obtained as described by Harden (11) for the preparation of zymin, were extracted with 12 volumes of distilled water at 5°C. After 30 min., the suspension was centrifuged at 6000 r.p.m. The supernatant fluid was adjusted to pH 7, and solid $(NH_4)_2SO_4$ added to 0.5 saturation. The precipitate was removed by centrifuging and more solid $(NH_4)_2SO_4$ was added to the supernatant to bring the latter to 0.8 saturation with respect to this salt. The resulting precipitate was separated and dissolved in water so that the volume was reduced to about 1/5 that of the original extract. This solution was then dialyzed 2–3 hr. in the cold. The dialyzed fluid contained about 50% of the total activity of the original extract. Such preparations could be stored frozen for several weeks without deterioration.

The ratio: glucose diphosphate/glucose formed was 0.01 with the crude extract and 0.15 to 0.20 with the final dialyzed solution after activation by cysteine (see below). Repeated fractionation by $(NH_4)_2SO_4$ precipitation resulted in extracts with a glucose diphosphate/glucose ratio of 0.35 to 0.50. Great losses of activity were encountered during this process of purification.

Activators and Inhibitors

The Action of Cysteine and Magnesium

Dialysis of the extracts against cold water resulted in a gradual reduction of their activity so that, at the end of 6 or more hours of dialysis, the activity might have disappeared completely. The addition of magnesium ion was tested because it activates many of the enzymes which act on phosphoric esters. As shown in Table II, no increase in

TABLE II

The Action of Cysteine and Magnesium Ions on the Formation of Glucose Diphosphate

Incubation of: 2 μM glucose-1-phosphate with 0.03 ml. purified enzyme 10 min. at 37°C. Total volume, 0.25 ml.

	μM Glucose diphosphate formed	Ratio: glucose diphosphate/glucose
Undialyzed enzyme	0.023	0.06
Dialyzed 4 hr.	0.015	0.02
Dialyzed + 1 μM Mg^{++}	0.015	0.02
Dialyzed + 1 μM cysteine	0.14	0.3
Dialyzed + 1 μM Mg^{++} + 1 μM cysteine	0.11	0.3

activity could be detected. However, when cysteine was added to the dialyzed extracts, the activity was increased 5- to 10-fold. On the other hand, the addition of cysteine had no effect on the liberation of reducing substances or inorganic phosphate. The optimum activating effect of cysteine was observed at a concentration of about 8×10^{-3} M.

The addition of boiled extracts of *E. coli* had no effect on the activity of the dialyzed enzyme.

The Action of Adenosine Phosphate

Glucose diphosphate has been found to be formed in muscle by transphosphorylation between glucose-1-phosphate and adenosinetriphosphate (12). The effect of the latter was investigated in the reaction as catalyzed by the extracts of *Escherichia coli.*

The effect of adenosinetriphosphate was tested at concentrations ranging from 10^{-5} to 10^{-2} *M*, and in no case could activation be detected. Concentrations higher than 10^{-3} produced an inhibition of the formation of glucose diphosphate. Moreover, the maximum amount of adenine compounds present in the enzyme was calculated from the extinction at 260 mμ and the results showed that the amount of glucose diphosphate formed was at least 10 times larger than the maximum amount of adenine compounds present. No acid-labile phosphate could be detected.

It does not seem likely that any reaction which leads to phosphorylation of the adenosine compound takes place under these experimental conditions. In some experiments, the reaction was allowed to take place in Warburg manometers at pH 7 in bicarbonate and a nitrogen-CO_2 gas phase. No acid formation was detectable and, therefore, reactions which would give rise to phosphorylation, such as the oxidation of glyceraldehyde or the formation of phosphopyruvic acid, could be excluded. These results appear to indicate that glucose diphosphate is not formed by transphosphorylation between ATP and glucose-1-phosphate.

Fluoride

Fluoride, at a concentration of 5.8×10^{-4} *M*, inhibited the phosphatase action to the extent of 75% and retarded the rate of glucose diphosphate formation to about the same degree.

Phloridzin

Phloridzin at a concentration of 3×10^{-4} *M* produced no appreciable change on the course of the reactions.

pH Optimum

The rate of formation of glucose diphosphate was found to be affected only slightly by fairly large changes in pH (Table III). The greatest activity was between pH 5 and pH 6 with acetate, maleate, or phosphate buffers.

Chemical Changes Produced by the Enzyme Preparation

The course of the reaction involving the conversion of glucose-1-phosphate to glucose diphosphate by the purified enzyme was similar to that by intact cells. There was first an increase in glucose diphosphate followed by its gradual disappearance. It has not been possible to separate the formation from the destruction of the diphosphate by purification of the enzyme. Fig. 2 illustrates the changes which occurred during the reaction. There was a gradual increase in inorganic phosphate and in the reducing power of the reaction mixture, coincident with a decrease in the acid-labile phosphate. At least 80% of the reducing substances were not precipitated by the $ZnSO_4$-$Ba(OH)_2$ reagent (13), which precipitates the hexose phosphates.

TABLE III

pH Optimum

Incubation of 2 μM of glucose-1-phosphate, 2 μM of cysteine, 0.1 ml. of 0.1 M of acetate buffer and 0.03 ml. of purified enzyme. Total volume, 0.35 ml., 15 min. at 37°C. Results in μM.

pH	3.8	4.4	4.7	5.3	5.9	6.2
Glucose diphosphate formed	0.08	0.15	0.17	0.23	0.19	0.15

These changes can be explained by the occurrence of the following reactions:

(a) 2 glucose-1-phosphate → glucose diphosphate + glucose,
(b) glucose-1-phosphate → glucose + inorganic phosphate,
(c) glucose-1-phosphate → glucose-6-phosphate.

Reaction (a) would be a transphosphorylation somewhat similar to the phosphoglucomutase reaction, which has been formulated (2) as follows:

(d) glucose-1-phosphate + glucose-1,6-diphosphate $\rightleftharpoons$ glucose-1,6-diphosphate + glucose-6-phosphate.

Reaction (b) might occur directly or with (a) or (c) as intermediates. As to reaction (c), it is relatively slow as compared with the phosphatase (b) and, since large amounts of glucose diphosphate are formed, it was not possible to test whether it takes place by mechanism (d). Another possible mechanism for the formation of glucose-6-phosphate would be the removal of the phosphate at position one of glucose diphosphate by phosphatase.

The Effect of Glucose

Addition of glucose produced striking changes in the course of the reactions (Table IV). Whereas the formation of glucose diphosphate was not appreciably affected, there occurred a great increase in the acid-stable phosphate and a decrease in the liberation of inorganic phosphate. The amount of fructose was also increased.

TABLE IV

The Action of Glucose

10 μM glucose-1-phosphate+0.3 ml. enzyme solution+ 10 μM cysteine. Total volume, 2 ml., 30 min. at 37°C. Results in μM. Values for fructose were corrected by subtracting the values found at t = 0.

Additions	Glucose diphosphate formed	P inorganic formed	Acid-stable P	Fructose
None	0.79	7.5	1.4	0.15
30 μM glucose	0.67	6.2	3.2	0.80
60 μM glucose	0.71	4.9	4.0	1.40

Sorbitol or ethanol did not produce an effect similar to glucose, whereas fructose was about half as effective at the same concentration.

The stable ester, which was formed in larger amounts in the presence of glucose, was presumably glucose-6-phosphate which was in equilibrium with fructose-6-phosphate. This explains the concomitant increase in the "fructose" content of the reaction mixture.

The action of glucose was similar to that described with liver enzymes (14), where it has been interpreted to be due to the inhibition of a specific phosphatase acting on glucose-6-phosphate.

The partially purified enzyme of *E. coli* was found to liberate inorganic phosphate, not only from the glucose phosphates but also from the α- and β-galactose-1-phosphates, α- and β-glycerophosphates, and phenylphosphate. Of these, phenylphosphate was the most rapidly hydrolyzed.

Glucose was found to inhibit also the liberation of inorganic phosphate from α-galactose-1-phosphate.

Discussion

The ready fermentability of glucose monophosphate by live *Escherichia coli* and related organisms reveals a difference with intact yeast cells, which do not measurably utilize phosphoric esters. Presumably, the cell membrane of *E. coli* is permeable to monophosphoric esters and, to a lesser extent, also to diphosphoric esters. Thus, during the fermentation of glucose-1-phosphate, the diphosphate is formed, passes to the medium, and is utilized at the end of the fermentation.

Glucose diphosphate has been found to be an intermediate in the utilization of glucose-1-phosphate, and this raises several problems, such as the mechanism of its formation and destruction and its role in the normal metabolism of *E. coli*.

The formation of glucose diphosphate appeared to take place by a mechanism different from that in animal tissues or yeast, where it has been demonstrated that glucose-1-phosphate is transphosphorylated by adenosinetriphosphate (12). Definite proof of the mechanism of synthesis of glucose diphosphate by *E. coli* must be deferred until the specific enzyme system involved can be separated from interfering systems. However, the evidence at hand can best be interpreted by assuming that the conversion of glucose-1-phosphate to glucose diphosphate involves a transfer of phosphate from position-1 of glucose-1-phosphate to position-6 of another molecule of the same substance.

The utilization of glucose diphosphate will require further investigation. The partially purified preparation which catalyzed its synthesis appeared to break it down mainly by the action of a contaminating phosphatase. Some experiments designed to detect in crude extracts an enzyme similar to aldolase, but which would act on glucose diphosphate, were not successful.

Summary

Glucose diphosphate was found to be formed by *Escherichia coli*, *Aerobacter aerogenes* and *Klebsiella pneumoniae* when incubated with glucose-1-phosphate. No formation was detected from other sugars or their derivatives. Living cells fermented glucose monophosphates and free glucose at about the same rate, and fructose diphosphate or glucose diphosphate hardly at all.

A partially purified enzyme was prepared which transformed glucose-1-phosphate into reducing substances and inorganic phosphate, with the transient formation of glucose diphosphate.

The formation of glucose diphosphate was activated by cysteine and inhibited by fluoride. Glucose decreased the rate of liberation of inorganic phosphate and increased the formation of acid-stable phosphoric esters.

It is postulated that glucose diphosphate is formed by transphosphorylation between two molecules of glucose-1-phosphate.

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Thermal Enzymes[1]

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Introduction

Certain organisms prefer high temperatures for growing. This fact has always presented somewhat of an anomaly. Proteins in general are inactivated at 55°C. The majority of enzymes are destroyed at that temperature. The logical consequence of the growth of thermophilic life is that the organism must have enzymes and proteins resistant to the usual effects of heat. Another possibility would be that the enzymes and proteins of the thermophiles are no different from the usual ones, but that these organisms contain protectors of the protein structure.

We have attacked the problems of high temperature life with the idea in mind of comparing the heat stability of the enzymes themselves with the heat stability of enzymes found in animal tissues and in microbial cells living at much lower temperatures. For the study we have chosen a stenothermophilic bacterium that grows at 65°C.

Gaughran (1) has shown that thermophilic bacteria metabolize many common substrates. Succinate, formate, glucose, lactate, and citrate were easily oxidized by whole resting cells. This in itself is important information, since it tells us that we can expect to find the common enzymes and not a group of different enzymes.

The results reported in this paper are mostly concerned with the enzyme malic dehydrogenase. This enzyme from the thermophile does,

[1] **The initial work on this problem was made possible through a grant from Mr. J. L. Welch of Omaha, Nebraska. The major part of the work presented in this report was financed by the U. S. Public Health Service.**

indeed, possess a heat stability that is not shared by the corresponding enzyme from a mesophilic bacterium and from kidney tissue.

EXPERIMENTAL

Growth and Harvest of Organisms

The bacterium studied is an organism obtained from the National Canners Association, isolate No. 2184, recovered from spoiled canned peas in June 1922. It is one of the "flat sour" types of spoilage organism.

Culture No. 2184 is a sporulating aerobic rod of the stenothermophilic type. Its optimum temperature is 65°C., its maximum 70°C., and its minimum 45°C. It grows sparsely at 50°C. On the usual carbohydrate media used for taxonomic studies, the organism produces no gas but produces acid only from maltose (slightly), glucose and xylose.

The medium in which the organism was grown for large masses of cells had the following composition: beef extract (Armour), 5.0 g.; peptone (Bacto), 3.0 g.; glucose, 1.0 g. These ingredients were dissolved in 1 liter of a mixture of $M/20$ NaOH and $M/20$ KH_2PO_4. The medium was distributed in 4.0 liter quantities and autoclaved at 15 lb. for 60 min., cooled to 65°C., and inoculated. The inoculum consisted of a 250 ml. broth culture, 24 hr. old. The pH after sterilization was 7.0.

Each 6 liter Erlenmeyer flask was then fitted with a sterile sintered glass sparger inserted through a large cork wrapped in nonabsorbent cotton. All operations were conducted under rigid asepsis.

The 6 liter flasks were placed in a 65°C. incubator and the spargers attached to a manifold which admitted compressed air. The air was rendered sterile by passage through sterile cotton filter chambers.

Growth curve studies indicated that the maximum viable cell population appeared after 22 hr. incubation at 65°C. Cells were generally harvested at 16 hr. and separated from the medium with a Sharples centrifuge. Few or no spores appeared in the broth cultures, as one would expect. The absence of spores was confirmed by the Dorner and Wirtz staining methods.

For comparing the enzymes of thermophilic organisms with mesophilic bacteria, cultures of *B. subtilis* were chosen (strains, Marburg C-4 and Lawrence and Ford S-8). The mesophiles were treated exactly as No. 2184 with the exception of the temperature of incubation which was 37°C. and the length of incubation extended to 24 hr.

The organisms obtained from the Sharples centrifuge were washed two or three times in buffer and then recentrifuged at 3400 r.p.m. The wet cell preparation was then spread into thin layers and dried *in vacuo* over P_2O_5. The weight of dried organisms from a single run of six 6 liter flasks usually amounted to about 3 g. When dried, the organisms had a red color.

Lysis of the Organisms

The lysozyme method of Utter, Krampitz and Werkman (2) was used. Lysates prepared by this method were quite viscous, a condition noted by Herbert and Pinsent (3) in the lysis of *Micrococcus lysodeikticus*.

Separation of the Red Fraction from the Lysate

When the lysate was mixed with an equal volume of $M/15$ phosphate buffer of pH 7.4 and centrifuged at a speed of 3400 r.p.m. for 1 hr., a bright red, semisolid layer separated. This layer could easily be removed with a syringe. By saturating the lysate with NaCl, better separation was obtained. The red fraction retained its color and activity for at least several days when stored in the cold at 4°C.

Heat Treatment of the Lysate and the Red Fraction

Approximately 1 ml. of the crude lysate was placed in a 13 × 100 mm. Pyrex test tube and immersed in a water bath at the required temperature for the various intervals. At the end of the intervals the tubes were immediately placed in cold water.

Because the red fraction was obtained in small amounts (usually 1–2 ml.) as a thick suspension, it was necessary to heat it in a single tube and to take aliquots at the desired intervals. For ease in pipetting into the Warburg vessels, the cooled aliquots were diluted with an equal volume of water.

Measurement of Enzyme Activity

All determinations were made at 37°C. in the Warburg apparatus. Oxygen consumptions for the various enzymes were determined by adding the following solutions to the flasks. Malate: 0.5 M sodium malate, 0.1 ml.; 0.05 M NaCN, 0.2 ml.; 1% methylene blue, 0.05 ml.; coenzyme I, 0.2 ml. of 1% solution, or trace of solid. Citrate: 0.1 M sodium citrate, 0.4 ml.; 0.01 M sodium arsenite, 0.1 ml. Succinate: 0.1 M sodium succinate, 0.3 ml.; cytochrome c (Viobin) 0.1 ml.; 0.004 M $CaCl_2$, 0.3 ml.; 0.004 M $AlCl_3$, 0.3 ml. α-Ketoglutarate: 0.01 M sodium α-ketoglutarate, 0.3 ml.; 1 M sodium malonate, 0.1 ml.; 0.2 M $MgSO_4$, 0.2 ml. Pyruvate: 0.1 M sodium pyruvate, 0.5 ml.; 0.1 M $NaHCO_3$, 0.2 ml. In addition to the above, 0.5 ml. of lysate (or 0.1 ml. of red fraction) was added to each of the vessels as well as 1.0 ml. of $M/15$ phosphate buffer, pH 7.4. Two-tenths ml. of 10 N NaOH was placed in the center well. Water was used to make the total volume 3.0 ml. A control containing all ingredients except the substrate was run in each case.

Kidney Homogenate

Two small rat kidneys weighing 1.0 g. total were homogenized in the Potter homogenizer with 1.5 ml. of 0.9% NaCl and 0.5 ml. of phosphate buffer of pH 7.4. This concentration of tissue was chosen to give approximately the same nitrogen concentration as found in the cell lysates. The kidney homogenate contained 8.8 mg. nitrogen/ml., and lysates contained 7.3–9.6 mg. nitrogen/ml.

The heat inactivation was conducted in the manner used with the red fraction.

Results

Whole cells of thermophile No. 2184 were able to oxidize malic, α-ketoglutaric, pyruvic, citric, and succinic acids at 37°C., even though this temperature is far below the growing temperature of the organism.

At times, the cell preparations oxidized all of the acids, but more often the results were high for only one of the substrates and not always for the same substrate. After about 12 runs we dropped the practice of screening whole cells for activity and went directly to the lysed cell preparations.

In comparison with the whole cells the enzyme activity of the lysed preparations was much better and, in general, more consistent with respect to the number of substrates oxidized. A typical run on lysates follows.

The lysates contained enzymes for malic, α-ketoglutaric, citric, pyruvic and succinic acids. Of these, malic dehydrogenase from different preparations was the most consistent, both in amount and activity. The succinic dehydrogenase was the least consistent.

TABLE I

Activity of Cell Lysates

Substrate	Oxygen uptake in µl./hr.		
	A[a]	B	C
Malate	43	176	139
Succinate	13	7	4
α-Ketoglutarate	2	21	5
Citrate	22	35	10
Pyruvate		21	15

[a] Letters indicate different cell harvests of organism No. 2184. Each assay was corrected by its own blank, generally 2–4 µl./hr.

For comparing heat stabilities of enzymes from various sources, we chose the malic dehydrogenase system. The lysates of the bacteria were prepared in such manner as to give high concentrations of total protein so that the original conditions of the cell might be paralleled as much as possible. The homogenates of the kidney tissues were made up to correspond to the nitrogen concentrations of the bacterial lysates. In this way, all enzymes were compared for heat-stability in solutions of approximately equal concentrations of protein.

The malic dehydrogenase system of the thermophile proved to be stable to destruction for 2 hr. at 65°C. At 75°C. it was slowly inactivated over a period of 90 min. At 80°C. the destruction was rapid and complete in a few minutes.

In contrast to the behavior of the thermophile enzyme was the lack of stability of the same enzyme obtained from a mesophilic bacterium. The malic dehydrogenase from the mesophile was inactivated in 10 min. at 65°C. The bacterium belongs to the same genus as the thermophile but differs in that it does not grow at 65°C. but, instead, grows at 37°C.

The malic dehydrogenase from rat kidney was even more sensitive to heat destruction at 65°C. than was the enzyme from the mesophilic bacterium. The rat kidney enzyme was completely destroyed in a few minutes.

The results on heat stability are summarized in Figs. 1 and 2.

In an effort to obtain the malic dehydrogenase in purer form we tried fractionating the lysates by centrifuging. The malic dehydrogenase

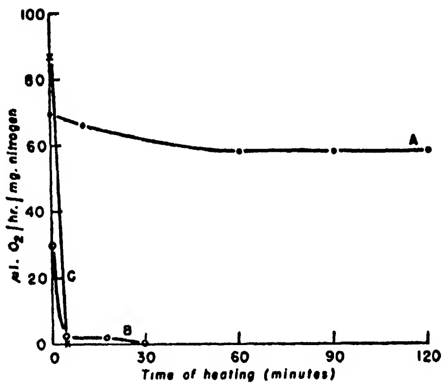

FIG. 1. Malate activity after heating at 65°C.; A, cell lysates of thermophile No. 2184; B, cell lysates of a mesophile; C, kidney homogenate.

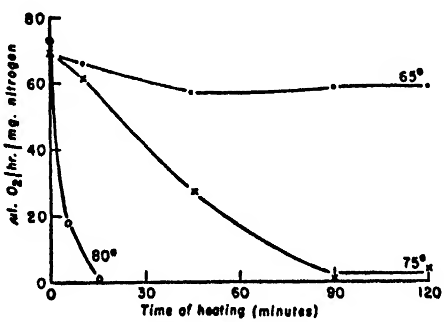

FIG. 2. Malate activity of cell lysates after heat treatment at different temperatures.

centrifuged down with an insoluble residue which we have called the "red fraction" because of its pink tinge. Almost all of the malic dehydrogenase activity resides in the red fraction. Table II illustrates the difference in activity between the red fraction and the crude lysates. It will be seen that the activity of the red fraction is about three times as great as that of the crude lysates.

TABLE II

Malate Activity of Red Fraction

	Oxygen uptake in µl./hr./mg. N	
	A[a]	B
Cell lysates	37	26
Red fraction	104	64

[a] Letters indicate different cell harvests of organism No. 2184.

The malic dehydrogenase of the red fraction proved to be heat-stable. If anything, it showed better heat stability than did the crude preparations. At 65°C. the activity did not diminish greatly over a 2 hr. heating period (Table III).

TABLE III

Stability of Red Fraction at 65°C.

Malate Activity

Time of heating *min.*	µl. Oxygen/hr./mg. N
0	95
5	83
15	78
30	68
120	57

Together with the usual preparation of malic dehydrogenase from mesophiles and animal tissues, a flavoprotein, diaphorase, accompanies the enzyme. Whether this is the case with the thermophile enzyme we do not know. Spectroscopic examination revealed no absorption bands for flavin pigments. Further, repeated washing of the red fraction with buffers did not reduce the malic activity or the red color.

The malic enzyme from the red fraction can use coenzyme I as a coenzyme (without coenzyme, 69 µl. oxygen/hr.; with, 157 µl. oxygen/hr.). This is not conclusive evidence that coenzyme I is the real coenzyme present in thermophiles but makes it highly likely.

As yet, we hesitate to speculate on the general significance of the heat stability of malic dehydrogenase from thermophile No. 2184. The heat data on the other metabolic enzymes of thermophiles must be obtained before any generalizations can be drawn. It does, however, seem worthy of note that the enzyme in this case is stable at the temperature preferred for growth by the bacterium. As soon as this temperature is exceeded, the organism not only stops growing but its malic dehydrogenase becomes susceptible to heat destruction. It may be very likely that enzymes of thermophilic life are constructed to stand the optimum growing temperature of the particular organism but not much more than that.

Of further significance to the problem of thermophilic life is the fact that we have thus far found no digestive enzymes produced by No. 2184. We have tested for amylase activity by the usual iodine method for α-amylase (4). No activity was ever found, although it must be stated that the organism grows well in the presence of glucose, and thus it might well be that the organism does not produce any diastatic enzyme. Proteolytic enzymes could not be found by the methods of Anson (5), nor by the formol titration method. Attempts to obtain a protein precipitate from the medium resulted in precipitates without activity.

Thus far, we have not found any mention in the literature of digestive enzymes in the medium for any thermophile after the regulation period of incubation at 65°C. or higher. Chopra (6) studied the proteolytic enzymes produced in the filtrates by the three thermophiles *Bacillus thermophilus*, *Bacillus aerothermophilus*, and *Bacillus thermoacidurans*. His temperature of incubation was 50°C. The difference between 50°C. and 65°C. is a very critical one in the stability of proteins to temperature inactivation. The discovery of digestive enzymes in the medium after an incubation of 65°C. or above would be of considerable significance, since here the enzymes would have been separated from the protective influences of the cell.

Summary

Cell lysates of a stenothermophilic bacterium oxidize malate, succinate, α-ketoglutarate, pyruvate, and citrate.

The malic dehydrogenase from the thermophile is stable to heat inactivation at 65°C., whereas the corresponding enzyme from a mesophile is inactivated in 10 min. at the same temperature.

A separation of a fraction called the red fraction was effected by centrifugation of the lysates. This red fraction contains the bulk of the malic dehydrogenase.

The malic enzyme from the red fraction can use coenzyme I as a coenzyme.

No amylases or proteinases were found in the filtrates from this thermophile.

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Dihydroxyphenylalanine Decarboxylase: Preparation and Properties of a Stable Dry Powder

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Introduction

An enzyme which decarboxylates L-3,4-dihydroxyphenylalanine (dopa) to 3,4-dihydroxyphenylethylamine (hydroxytyramine) was discovered by Holtz, Heise and Lüdtke (1) in extracts from the kidneys of guinea pigs and rabbits. Subsequently, this enzyme was found to occur also in the kidneys of other species, in liver (2,3), in the intestinal tissue of guinea pigs (4) and in pancreas from cats, guinea pigs, dogs and pigs (5). A list of the dopa decarboxylase activity of various tissues compiled by Blaschko (6,7), shows that guinea pig kidneys are a particularly rich source for this enzyme. Page (8) attempted to prepare stable dry powders from kidney extracts and stated: "drying in the cold by a lyophile process reduced the activity of rabbit kidney extracts by at least 50%, and abolished the activity of guinea pig extracts on two occasions." Drying of saline extracts in cellophane sacs at room temperature yielded stable preparations, but the reduction in activity during the drying process was "quite variable" (8).

This report describes the preparation of dopa decarboxylase as a stable dry powder and some properties of this material. Experiments with dopa decarboxylase were usually carried out at pH 7.4 (1,6,8), but Holtz and Credner (5) observed higher activity at pH 6.5 as early as 1942. Lack of a systematic investigation of the influence of pH on the activity of this enzyme prompted us to determine its pH-activity curve.

Experimental

1. Determination of Activity

The decarboxylation of dopa was followed manometrically with the Warburg apparatus at 37°C. The main vessel contained 4 ml. enzyme solution ($M/15$ phos-

phate buffer) and air was displaced by nitrogen to avoid confusing side reactions. After temperature equilibrium had been reached, 2.2 mg. L-dopa (250 μl. CO_2) in 0.5 ml. water were added from the first side vessel. Readings were taken at 10 min. intervals and the reaction was terminated, usually after 60 min., by the addition of 0.5 ml. 1.2 *N* H_2SO_4 from the second side vessel. The individual readings were corrected for retained CO_2, liberated by the addition of acid. A blank contained water instead of substrate and was used to correct for small amounts of CO_2 contained in the buffer or formed by the kidney extracts in absence of dopa.

2. *Preparation of Stable Dry Powders*

In a Waring Blendor, 30 g. fresh kidneys were mixed with 100–120 ml. ice cold water for 4 min. at room temperature. The material was then placed in a refrigerator for 1 hr. and later centrifuged for 15 min. (size 2 centrifuge, conical head, 4400 r.p.m.). The pink and turbid supernatant fluid was decanted, frozen rapidly and dried *in vacuo* from the frozen state. The weight of dry powder obtained from guinea pig kidneys was regularly 10% of that of the fresh kidneys; the yield from rabbit kidneys varied from 10–14 g./100 g. fresh kidneys. The dried material was stored *in vacuo* over $CaCl_2$ at room temperature (23–25°C.). The decarboxylase activities of the dry powders (dissolved in *M*/15 phosphate buffer, pH 6.8) were found to be 75% (rabbit) and 45–60% (guinea pig) of those observed with phosphate buffer extracts from fresh kidneys of the same batch. As customary, activities were calculated from the volumes of CO_2 liberated during the first 10 min. after addition of substrate and expressed in terms of Q_{CO_2} (μl. CO_2/hr./mg. fresh tissue). An extract from fresh rabbit kidneys, for example, gave a Q_{CO_2} of 0.91 and yielded a dry powder with a Q_{CO_2} of 0.68. Calculated per mg. of dry powder instead of fresh kidneys, the activities of several preparations from rabbits varied between 3.7 and 4.9, but decreased on standing over $CaCl_2$ *in vacuo*. During a storage period of 6 weeks, for example, the activity of one of these preparations dropped from 3.72 to 1.98 (47% loss). Several extracts from guinea pig kidneys had a Q_{CO_2} of 2.0–2.5 and the corresponding dry powders gave a Q_{CO_2} of 1.0–1.2 (per mg. fresh kidney) or 10.3–12.3 (per mg. dry powder). The material from guinea pig kidneys was relatively stable. A product with a Q_{CO_2} of 11.1 on the day of preparation retained its activity for 4 months, giving a Q_{CO_2} averaging 11.0 in 9 determinations performed after 100–120 days of storage. Longer storage over $CaCl_2$ *in vacuo* led to a decrease in activity and the Q_{CO_2} values after 6.5 and 8 months of storage were 7.7 and 5.4, respectively.

The amounts of dry powders used in these stability studies were 25–60 mg. (guinea pigs) and 90–120 mg. (rabbits) per Warburg flask, and liberated between 35 and 85 μl. CO_2 during the first 10 min. after addition of substrate.

3. *pH-Activity Curve*

The activity of dopa decarboxylase was tested through the pH-range 5.5–8.0. Each Warburg flask received 4 ml. *M*/15 phosphate buffer (of a selected pH) containing 30 mg. (guinea pig) or 40–80 mg. (rabbit) dry powder. All pH measurements in the enzyme-buffer mixtures were made using a glass electrode, and in each set of experiments there was included an assay at pH 7.07, to permit corrections, if necessary, for small changes in enzyme activity from one group of tests to the next. The volumes of

CO_2 liberated during 60 min. were expressed as per cent of the amount obtained at pH 6.80, which was found to be the pH for optimal activity. The relationship between pH and activity is shown in Fig. 1.

4. *Effect of Pyridoxal Phosphate on Dopa Decarboxylase Activity*

Three hundred mg. dry powder (guinea pig) were dissolved in 30 ml. $M/15$ phosphate buffer, pH 6.81, and dialyzed against 1000 ml. of the same buffer for 55 hr. at 4°C. The enzyme solution was then tested for activity. A volume containing 30 mg. powder formed before dialysis 130 μl. CO_2/hr. and after dialysis 106 μl. CO_2/hr. When a solution of dry powder in buffer was stored for 70 hr. at 4°C. instead of being dialyzed

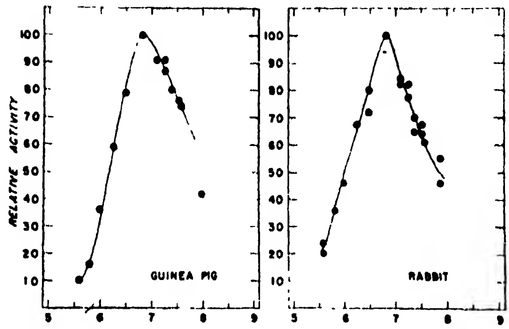

FIG. 1. Influence of pH on the activity of dihydroxyphenylalanine decarboxylase from guinea pig and rabbit kidneys. Activities expressed as per cent of optimal activity. Incubations carried out for 60 min. at 37°C. in $M/15$ phosphate buffers. Substrate concentration: 2.2 mg. L-dopa (250 μl. CO_2) in 4.5 ml. incubation mixture.

a volume containing 30 mg. dry powder produced only 98 μl. CO_2/hr. under strictly comparable conditions. The addition of 0.13 mg. barium pyridoxal phosphate (9) to each incubation mixture nearly doubled enzymic activity. The amounts of CO_2 produced per hr. were then 241 μl. (fresh, undialyzed), 213 μl. (dialyzed for 55 hr.) and 200 μl. (stored for 70 hr. at pH 6.81 and 4°C.).

5. *Reaction Kinetics*

The time-activity curve for the enzymic decarboxylation of dopa did not follow the course of a first order reaction. In an evaluation of our experimental data using the equation $k = 1/t \log (a/a - x)$, it was found that the numerical values for k at 60 min. were only about 1/2 of those calculated for the initial 10 min. period. Similar observations had been made already (10) in studies with glutamic acid decarboxylase of higher plants, and it was reported that the addition of pyridoxal phosphate resulted in a first order reaction curve for this enzyme. With dopa decarboxylase, however, this change to a first order curve did not occur, although the addition of pyridoxal phosphate lessened the extent of the decrease in k during a 60 min. reaction period.

The investigation of glutamic acid decarboxylase had revealed the existence of a linear relationship between reaction velocities (x/t = µl. CO_2/min.) and the amount of work already performed by the enzyme (x = µl. CO_2 liberated at time t). Examination of the data obtained with dopa decarboxylase showed again that the reaction velocities decreased as a linear function of the extent of substrate decomposition. The equation for the straight lines obtained on plotting x/t against x (10) may be written as:

$$x/t = m \cdot x + b$$

and can be rearranged to read:

$$1/t = b \cdot 1/x + m.$$

This is the equation for a straight line obtained when $1/x$ is plotted against $1/t$ (see Fig. 2).

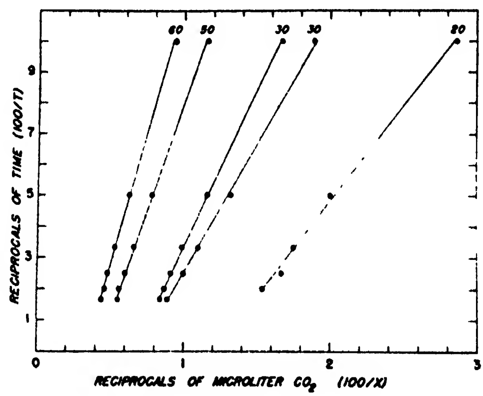

FIG. 2. Graph showing the linear relationship between the reciprocals of time and the amounts of CO_2 formed by dopa decarboxylase from guinea pig kidneys. The numbers on the curves refer to the mg. of dry powder used in 4.5 ml. incubation mixture at pH 6.80 and 37°C. The data were obtained using several different dry powders. Substrate concentration: 2.2 mg. L-dopa (250 µl. CO_2). Initial velocities are given as the slopes of the lines.

For any given observation period, there was a linear relationship between enzyme concentration (mg. dry powder/4.5 ml. incubation mixture, containing 2.2 mg. dopa) and amount of CO_2 produced. Data obtained with guinea pig kidneys (dry powder) illustrate this relationship in Fig. 3.

6. *Inhibition of Dopa Decarboxylase*

a. Hydroxylamine. An aqueous solution of hydroxylamine hydrochloride was adjusted to pH 6.8 with NaOH and diluted with 9 vol. $M/15$ phosphate buffer (pH 6.80). The incubation mixtures contained in the main vessel 50 mg. dry powder (guinea pig) in 3 ml. buffer and 1 ml. inhibitor solution. In the control experiment,

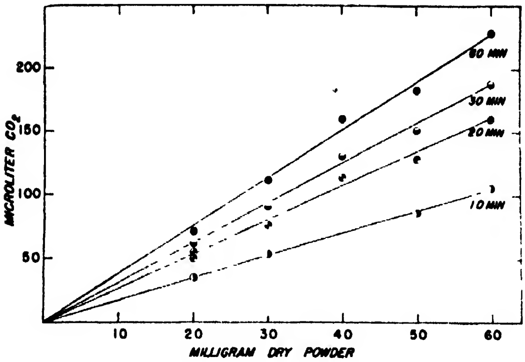

FIG. 3. Linear relationship between CO_2 production and dopa decarboxylase concentration. Dry powders from aqueous extracts of guinea pig kidneys were used. Substrate concentration: 2.2 mg. L-dopa in 4.5 ml. incubation mixture, pH 6.80, 37°C.

1 ml. buffer was substituted for the hydroxylamine solution. Typical results are shown in Table I. In addition to its inhibitory activity, hydroxylamine had a striking effect on the time-activity curve of the enzyme. The reaction velocities remained fairly constant during a 60 min. period, in contrast to observations in absence of inhibitor and suggested a zero order reaction course.

b. Pteridine Derivatives. The following substances were tested for inhibitory activity: 7-methylfolic acid (I), 2,4-dihydroxy-6,7-dimethylpteridine (II), 2-amino-4-acetamido-6,7-diphenylpteridine (III), 2,4-diamino-6,7-bis-(*p*-sulfinomethylaminophenyl)-pteridine disodium salt (IV), and 2-amino-4-hydroxypteridine aldehyde-(6) (V).

TABLE I

Inhibition of Dopa Decarboxylase by Hydroxylamine

Time	Hydroxylamine concentration (m*M*/l.):				
	Zero	0.05		0.10	
	µl. CO_2	µl. CO_2	Inhibition	µl. CO_2	Inhibition
min.			*per cent*		*per cent*
10	86	24	72	17	80
20	128	50	61	34	73
30	151	77	49	53	65
40	167	92	41	74	56
50	178	113	37	94	47
60	183	134	27	107	42

The results may be summarized as follows:

Compound	Concentration in millimole/l.	Per cent inhibition (60 min.)
I (11)	0.5	16
	2.5	43
II (12)	0.5	zero
III (13)	0.6	zero
IV (13)	0.4	47
V (14,15)	1.2	14
	2.3	32

There was little difference in the degree of inhibition caused by pteridine derivatives when readings after 10 min. and after 60 min. reaction time were compared. The various pteridines did not modify the time-activity curves in the manner observed with hydroxylamine.

Discussion

The stability of dry powders prepared from aqueous extracts of guinea pig kidneys facilitates the investigation of dopa decarboxylase. Using this material, a number of data characterizing this enzyme were obtained which, heretofore, had been unavailable. The pH-activity curve shows that decarboxylation occurred with maximal speed at pH 6.80 with dry powders from guinea pig or rabbit kidneys. As can be seen from this curve, previous investigators, working at pH 6.5 or 7.4, utilized only about 80% of the potential enzymic activity.

Dopa decarboxylase lost activity on standing in phosphate buffer solution and this process was not accelerated by dialysis. This observation, together with results obtained by Blaschko (6), who noticed no decrease in activity of pig kidney extracts on dialysis for 40 hr., speaks against the removal by dialysis of a prosthetic group in dopa decarboxylase. Green *et al.* (16) inactivated dopa decarboxylase by dialysis against $M/500$ NH_4OH and restored some activity by the addition of pyridoxal phosphate and cysteine. There were, however, considerable irreversible losses. No data on the effect of ammonia without dialysis are given and it is, therefore, questionable whether dialysis contributed anything in this inactivation procedure. Our finding that the addition of pyridoxal phosphate doubled the activity of dopa decarboxylase, regardless of whether the enzyme had or had not been inactivated to some extent prior to this addition, points to the presence of an excess of apoenzyme in our preparations. This excess may very well be the result of a partial destruction of pyridoxal phosphate during the

preparation of enzyme dry powder from kidneys. The effectiveness of pyridoxal phosphate lends support to the view that this member of the vitamin B_6 group forms the active center of dopa decarboxylase. Further evidence in this direction has been presented recently by Blaschko *et al.* (17), who found that the addition of pyridoxal plus ATP raised the dopa decarboyxlase activity of liver extracts from pyridoxine-deficient rats.

There are obvious similarities between the reaction kinetics of dopa decarboxylase and of glutamic acid decarboxylase. The rapid decrease in enzymic activity during a 60 min. reaction period leading to a linear relationship between reaction velocities and degree of amino acid decarboxylation, is characteristic for both decarboxylases. Both enzymes were inhibited by hydroxylamine, as would be expected from the presence of an aldehyde group in their active center. There is a difference, however, in the effect of this inhibitor on the time-activity curve of the two decarboxylases. Only dopa decarboxylase showed a change towards a zero order reaction curve.

The inhibition of dopa decarboxylase by 7-methylfolic acid was first described by Martin and Beiler (18,19), who used extracts from rat kidneys in their assay procedure. The low decarboxylase activity of such extracts, however, does not permit an accurate determination of the degree of inhibition. Martin and Beiler based their calculations on differences of less than 4 μl. CO_2 (with 14.8 and 11.0 μl. CO_2 being liberated during 15 min.) and did not correct for retained CO_2 (pH 6.5). In the inhibition studies described here, 45-60 mg. dry powder (guinea pig) were used and produced 130–180 μl. CO_2/hr. or 75–100 μl. CO_2/15 min. in absence of inhibitor. As was to be expected, there are, therefore, differences between the results obtained by Martin and Beiler (18,19) and the data shown in this report. Martin and Beiler found that 7-methylfolic acid caused 25% inhibition in a concentration of 0.066×10^{-3} M/l. and 50% inhibition with a 10-fold increase in inhibitor concentration. We observed 16 and 43% inhibition with 0.5 and 2.5×10^{-3} M/l., respectively. The water-soluble sulfoxylate derivative of 2,4-diamino-6,7-bis-(p-aminophenyl)-pteridine was the most active inhibitor of the pteridines available to us, causing 47% inhibition in a concentration of 0.4×10^{-3} M/l. The reason for the inhibitory activity of pteridine derivatives on dopa decarboxylase is unknown. Folic acid does not seem to be a part of the decarboxylase molecule, and there is no correlation between the antifolic acid activities of

the compounds tested in this investigation and their effectiveness against dopa decarboxylase. Compound III, for example, was 165 times more powerful than compound IV as folic acid displacing agent in *Strep. faecalis* assays (13), but compound III did not inhibit dopa decarboxylase.

ACKNOWLEDGMENTS

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SUMMARY

1. A method is described for the preparation of dopa decarboxylase as a dry powder from aqueous kidney extracts. The material from guinea pig kidneys showed unchanged activity for a period of 4 months when stored over $CaCl_2$ *in vacuo*.

2. The pH-activity curve of dopa decarboxylase was determined. Preparations from guinea pig and rabbit kidneys showed optimal activity at pH 6.80.

3. The enzyme lost activity on standing in phosphate buffer solution. This process was not accelerated by dialysis.

4. The addition of pyridoxal phosphate to dopa decarboxylase solutions (fresh, stored, or dialyzed) doubled enzymic activity.

5. The time-activity curve for the enzymic decarboxylation of dopa did not follow the course of a first order reaction. Enzymic activity decreased rapidly with time and there was a linear relationship between reaction velocities and the extent of substrate decomposition.

6. The amount of CO_2 liberated during a given period of time was found to be a linear function of the amount of enzyme present.

7. Dopa decarboxylase was inhibited by hydroxylamine. In presence of this inhibitor, the reaction velocities remained fairly constant throughout a 60 min. period and suggested a zero order reaction course.

8. A variety of pteridine derivatives inhibited dopa decarboxylase. No correlation was found between the inhibitory effect of these substances and their folic acid-displacing activity in *Strep. faecalis* assays.

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Neomycin, Recovery and Purification [1,2]

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Introduction

Waksman and Lechevalier (1) have described neomycin as a substance produced by a culture of a *Streptomyces* (No. 3535), closely related to *Streptomyces fradiae*, when grown under shaken conditions in a peptone-glucose-meat-extract medium. This substance was obtained by the elution with formic acid in aqueous methanol of a Norit adsorbate of the active principle produced by the culture. Evidence, submitted below, points to the fact that this concentrate was not a single entity and should properly be referred to as the "neomycin complex." Waksman *et al.* (1,2,3,4) have discussed the production and antimicrobial activity of the "neomycin complex," briefly referred to as "neomycin." It is the purpose of this paper to describe methods of isolation and purification of this neomycin.

Experimental

Production of Neomycin

Neomycin was produced in essentially the same manner as streptothricin VI (5). The culture *Streptomyces* 3535 was maintained on potato dextrose-agar, which gave abundant growth of a well-sporulating culture. The inocula were prepared by washing the spores from small Blake bottle cultures and inoculating 100 ml. portions of a medium in 250 ml. Erlenmeyer flasks with 1 ml. of the spore suspension. The flasks were incubated at 26–28°C. on a rotary shaker at 240 r.p.m. for 16–72 hr. The contents of one flask were used to inoculate either 500 ml. in a 2 l. Erlenmeyer flask, with special indentations to serve as baffles, or 1000 ml. of the same medium in an ordinary 2 l. Erlenmeyer flask. These flasks were shaken at 180–200 r.p.m. at 26–28°C. for 120 hr.,

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the highest antibiotic activity being attained at that time. The age of the inoculum had no effect on the final potency of the culture filtrates.

Two media were used for the production of neomycin:

1. Bacto peptone 0.5%, glucose (cerelose) 0.5%, meat extract 0.5%, NaCl 0.5%, and tap water.
2. Soya peptone 2.0%, glucose (cerelose) 1.0%, meat extract 0.5%, NaCl 0.5%, and tap water.

The final pH for both media after autoclaving was 7.2-7.4.

When the first medium was used, the potency of the culture filtrates averaged 75 units/ml.; the average potency of the second medium was 100 units/ml. The increase in antibiotic potency in the soya peptone medium was apparently due to the disproportionate production of an antifungal factor, referred to in this paper as "Factor X."

Studies to determine the effect of the relative concentrations of glucose and peptone in the medium indicated that a higher ratio of peptone to glucose favored the production of Factor X; frequently as much as 30% of the activity was due to this factor, when 2% peptone was used. This effect was more pronounced, at ratios greater than unity, in the bacto peptone broths than in the soya peptone broths; the reverse was true when ratios less than one were used. The effect of increased aeration on the relative production of neomycin and Factor X, in a given medium, was studied by using the 2 l. baffle flasks (high aeration) and plain Erlenmeyer flasks noted above. For the bacto peptone medium, the percentage of Factor X increased from 25.2 to 66.6 when less air was supplied during growth of the organism; the respective percentages for the soya peptone medium were 40.0 and 69.2.

Assay of Neomycin

Assays were made by the agar dilution method (6) and by the agar diffusion or cup method (7). A unit of neomycin is the minimum amount of the antibiotic that will completely inhibit the growth of *Escherichia coli* A. T. C. C. 9637 in 1 ml. of nutrient agar. A sample of neomycin which had been assayed at 150 units/mg. by the agar dilution method was taken as the standard for the cup assay, with *Bacillus subtilis* A. T. C. C. 6633 as the test organism. The nutrient agar medium had the following composition: meat extract 0.3%, peptone 0.5%, NaCl 0.5%, agar 1.5%, and tap water. The final pH was 7.0-7.2. All samples and standard preparations were diluted with 0.1 *M* phosphate buffer at pH 7.0—7.2. When the logarithm of the concentration was plotted against the diameter of the zone of inhibition, as determined by the agar diffusion method, 2 different curves were obtained for neomycin and streptomycin, as shown in Fig. 1. Since the slopes of these curves were quite different, it was not possible to assay neomycin preparations against a streptomycin standard. Thus, a sample of neomycin diluted 1/10 and 1/50 gave potencies of 2,200 and 2,225 neomycin units/ml., whereas the respective values with streptomycin as the standard were 940 and 1500 units/ml.

In the assay procedure, when the agar medium and the buffer were adjusted to pH 8.0, the inhibition zone was much larger than when pH 7.0 was used. At pH 7.0, 15.3 units/ml. of neomycin gave an inhibition zone of 18.3 mm., and at pH 8.0 the same concentration of neomycin gave an inhibition zone of 24.4 mm. Higher assay values

were also obtained when the higher pH was used; for example, a culture filtrate that assayed 131 units/ml. at pH 7.0 assayed 208 units/ml. at pH 8.0.

Concentration of Neomycin from Broths

Crude preparations of neomycin hydrochloride were obtained from broths by 2 related methods:

The first was similar to the procedure used by Hutchison, Swart and Waksman (5) for streptothricin VI, except that the amount of Decalso was increased to 1 g. for 6000 units of neomycin. In a typical run, 3.2 l. of soya peptone broth, containing 601,600 units, was used as the

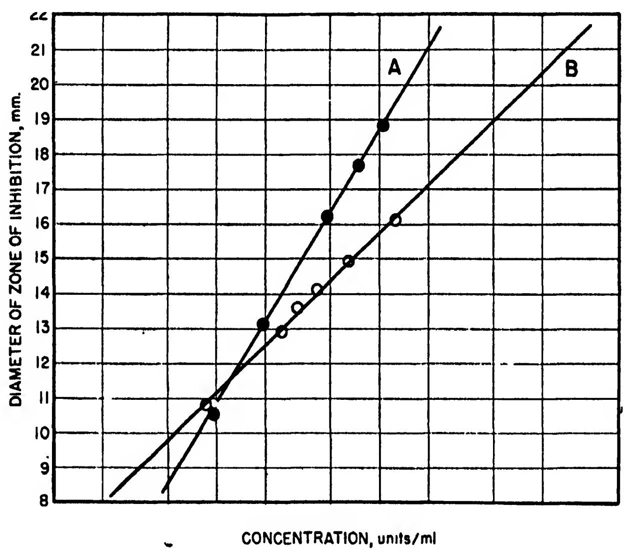

FIG. 1. Standard curves for streptomycin and neomycin. A—streptomycin; B—neomycin.

starting material. The filtrate from the acid-Darco clarification contained 470,000 units, representing a loss of 21.8% activity. Table I lists the pooled fractions obtained by the elution of the Decalso (80 g.) adsorbate contained in a column of 14 mm. inside diameter. Fractions 1, 2–6, and 7–9 were pooled, brought to pH 7.0 by addition of NaOH, and stirred 30 min. with Darco G-60 (100 g./l.); 32% of the activity was adsorbed. The filtrate was again adjusted to pH 7.0 and stirred a second time with Darco G-60 (100 g./l.); 5% of the original activity of the pooled eluates remained unadsorbed. The first adsorbate was eluted

twice, and the second adsorbate once, with 0.05 *N* HCl in 50% methanol (5 ml. of eluent/g. of Darco G-60). Each eluate was passed through an Amberlite IR-4B column to raise the pH without increasing the salt concentration, concentrated *in vacuo* at 30–35°C. to remove methanol, and lyophilized. The first eluate of the first adsorbate yielded 1,056 mg. of a solid having a potency of 137 units/mg., whereas 110 mg. of a solid with a potency of 41 units/mg. was obtained from the second eluate; these represented 30.8% and 1%, respectively, of the activity originally adsorbed on the Decalso. The eluate of the second adsorbate yielded 1080 mg. of a solid of a potency of 70 units/mg., representing 16.1% of the activity. The total recovery of activity from that adsorbed on the Decalso was 47.9%; the overall recovery from the original broth was 37.4%.

TABLE I

Elution of Decalso Adsorbate with 10% NH_4Cl

Fraction no.	Volume	pH	Assay	Activity recovered from	
				Darco filt.	Orig. broth
	ml.		*units/ml.*	*per cent*	*per cent*
1	10	6.8	1670	3.5	2.8
2–6	65	6.8–6.9	3050	42.2	33.0
7–9	75	6.9–7.0	2650	42.3	33.0
10	25	7.0	665	3.5	2.8
11–14	100	7.0	228	4.9	3.8
15	25	6.94	93	0.5	0.4
16–20	125	6.9–7.0	56	1.5	1.2
21	25	6.86	34	0.2	0.1
Totals	450			98.7	77.1

The second method used for the concentration of the "neomycin complex" as the hydrochloride, was similar to the methods used for the isolation of streptomycin and streptothricin. The broth was first clarified with activated charcoal at pH 2.0–2.5, as in Method 1. The neutralized filtrate was stirred 30 min. with Darco G-60 (20 g./l.) and filtered by suction. It is interesting to note here that neomycin is not as readily adsorbed on activated charcoals as are streptomycin and streptothricin. The adsorbate was washed with water on the funnel, transferred to a round-bottom flask, and stirred 30 min. with 95%

ethanol (10 ml./g. of Darco); the alcohol wash removed much pigment, without removing activity. The adsorbate, after drying overnight in air, was eluted twice with 0.05 *N* HCl in 50% methanol (5 ml. eluent/g. Darco). The eluates were passed separately through an Amberlite IR-4B column to raise the pH, concentrated *in vacuo* at 30–35°C. to remove methanol, and lyophilized. Solids having potencies of 52 and 56 units/mg. were obtained by this method from eluates 1 and 2, respectively, which were derived from 15 l. of a soya peptone broth having an activity of 102 units/ml.; the overall yield was 35%. An additional 10% of the original activity was eluted from the Darco adsorbates by stirring 30 min. with 0.1 *N* HCl in water saturated with *n*-butanol. Concentration *in vacuo* at 45°C., with subsequent freeze-drying, yielded a solid having a potency of 5 units/mg.

Purification of Neomycin by Precipitation with Picric Acid

The method of Peck *et al.* (8) for the purification of streptothricin by precipitation with picric acid has been used for the purification of neomycin. Picric acid (250 mg.), dissolved in 6 ml. of hot water, was added to 200 mg. of neomycin hydrochloride (111 units/mg.). The mixture was warmed until completely dissolved. On cooling to room temperature, a red oil separated out. After standing 5 hr. at 5°C., the supernatant liquid was decanted from the oil. The residue was dissolved in 3 ml. of hot methanol, excess HCl was added to convert the picrate to the hydrochloride, and about 10 vol. of ether used to precipitate the hydrochloride. The latter, redissolved and reprecipitated 3 times in this fashion, was obtained only as an oil. The supernatant was decanted, and ether removed from the residue, which was dissolved in water and lyophilized. A solid having a potency of 212 units/mg. was obtained in 24% yield.

Chromatographic Purification of Neomycin with Darco G-60

A mixture of 2 parts of Darco G-60 to 1 part of Hyflo Supercel was used as packing for a column. The adsorbent was wetted with methanol by sucking the solvent through the column at a rate of 2 ml./min. A saturated solution of neomycin hydrochloride in methanol was introduced into the column. The column was developed with methanol; 10 ml. fractions were collected. The method of Peck *et al.* (8), for testing with acetone to find active fractions, was used. The combined

TABLE II

Effect of Reaction and Temperature upon the Stability of Neomycin

Maximum time at which no loss of potency was observed

pH 2.0			pH 7.05			pH 9.0		
5°C.	25°C.	100°C.	5°C.	25°C.	100°C.	5°C.	25°C.	100°C.
7[a] days	2 days	1 hr.[a]	7 days[a]	1 day	1 hr.[a]	1 day	1 day	1 hr.[a]

[a] The stability of the neomycin preparation is greater than indicated by these results since these were the minimum time periods used in the test.

active fractions were treated with acetone to precipitate neomycin as the hydrochloride. The material was centrifuged, washed with acetone, and dried *in vacuo*. The preparation (940 mg., 70 units/mg.) was quantitatively converted, by chromatographing on 9 g. of the Darco-Hyflo mixture in a column of 14 mm. inside diam., to 576 mg. of a hydrochloride having a potency of 112 units/mg. The latter was converted in

TABLE III

Removal of Neomycin by Different Adsorbents

	Assay	Loss in potency	
	units/ml.	*units/ml.*	*per cent*
Control	44		
Norit A	16	28	63.6
Norit H 15	34	10	22.7
Norit AGNC	17	27	61.4
Nuchar C-190-N	4	40	90.9
Nuchar C-145-N	4	40	90.9
Nuchar C-145-Alk	4	40	90.9
Nuchar C-145-A	5	39	88.6
Darco G-60	9	35	80.0
Neutrol CH (A super filtrol)	3	41	93.2
Amberlite IR-4B	45	0	0
Amberlite IR-100H	22	22	50.0
Decalso	18	26	59.1
Zeo Karb H	3	41	93.2
Alumina	26	18	40.9
Celite 535	44	0	0
Celite 545	45	0	0
Hyflo Supercel	45	0	0

80% yield to a solid having a potency of 140 units/mg. by chromatographing on 9 g. of Darco-Hyflo mixture in the same fashion.

Stability of Neomycin

Neomycin is fairly stable over the pH range 2.0–9.0, as shown in Table II. For each pH tested, 50 mg. of neomycin hydrochloride, 100 units/mg., was dissolved in 40 ml. of water. The pH of the solutions was adjusted to 2.1, 7.05, or 9.0. Two ml. aliquots were pipetted into graduated centrifuge tubes and placed in a boiling water bath; samples were taken for assay, the volume being adjusted back to 2 ml., every 15 min. for the first hour, after which the 100°C. experiment was discontinued. The remainder of the solution was divided into two equal parts, one of which was placed in a rubber-stoppered test tube in a refrigerator at 5°C., and the other kept at room temperature. Samples were pipetted from the test tubes every 15 min. for the first hour, then at 2, 4, 6, and 24 hr., and at 24 hr. intervals thereafter for one week.

Adsorption of Neomycin on Common Adsorbents

One per cent Hyflo Supercel was stirred into a soya peptone broth, which was filtered by suction through a thin pad of Hyflo. There was no loss of activity. Fifty ml.

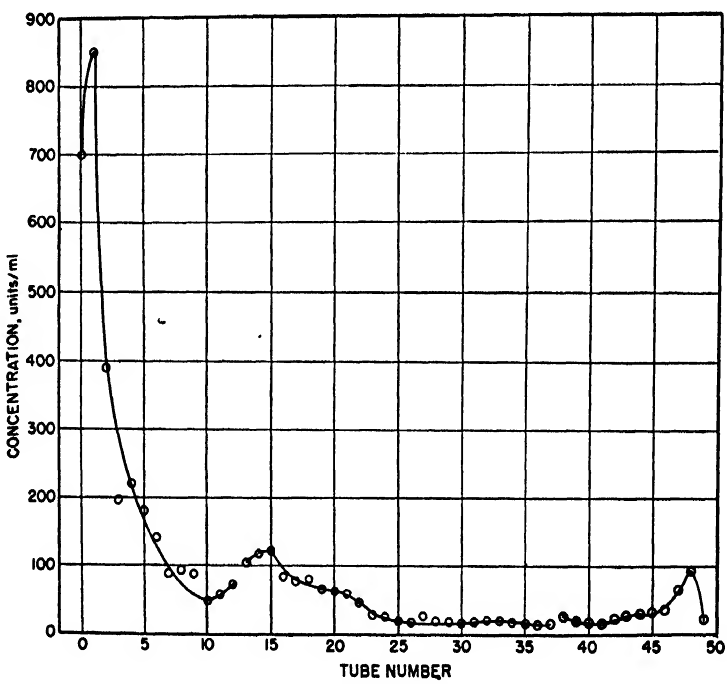

FIG. 2. Distribution curve for a 49 transfer of neomycin between borate buffer and pentasol at pH 7.3.

aliquots were mixed in 250 ml. Erlenmeyer flasks, at the pH (7.9) of the culture filtrate, with 1 g. of the adsorbents listed in Table III. These were shaken, along with a control containing no adsorbent, for 30 min. on a rotary shaker at 240 r.p.m., filtered and the filtrate assayed. Loss in potency was assumed to be due to adsorption.

Counter-Current Distribution Studies on the Neomycin Complex

A hydrochloride of the neomycin complex (27 mg., 150 units/mg.) was distributed in the Craig 24-plate counter-current distribution machine (9), using the borate buffer system at pH 7.6 described by Swart (10). This distribution curve showed a peak at tube 21, corresponding to a calculated distribution coefficient of 6, for the substance predominantly present in the original solid. The irregularity of the curve, though, indicated the possible presence of at least 2 other substances. To determine whether more than one substance was present, 600 mg. of a hydrochloride having a potency of 76 units/mg. was distributed in the borate buffer system at pH 7.3 (10), using the method of alternate withdrawals (11) to make 49 transfers. The distribution curve (Fig. 2) shows that at least 3 substances were present in the original solid. Table IV lists the antibacterial activity of these 3 fractions and the ratio of this activity to that of *E. coli* W. To demonstrate that neomycin is different from streptomycin and streptothricin, a distribution curve (Fig. 3) for a mixture of equal units of the 3 antibiotics was distributed in the borate buffer system at pH 7.6 (10).

TABLE IV

Antibacterial Spectrum of Fractions Obtained in a 49 Transfer Countercurrent Distribution of Neomycin

I = fraction of low distribution coefficient.
II = fraction of medium distribution coefficient.
III = fraction of high distribution coefficient.

Test organism	Dilution units/ml.			Ratio $\frac{\text{Specific activity}}{\text{Activity against } E.\ coli}$		
	I	II	III	I	II	III
E. coli W	150	150	20	1	1	1
B. subtilis	>1000	>1000	200	>6.7	>6.7	10
B. cereus	700	500	50	4.6	3.3	2.5
Bodenheimer	150	70	10	1	0.5	0.5
S. paradysenteriae	100	50	10	0.7	0.3	0.5
S. aureus	>1000	400	70	>6.7	2.6	3.5
Mycobacterium 607RN[a]	40	30	80	0.3	0.2	4

[a] Neomycin resistant.

Concentration of the Antifungal Factor X

Thirty-two l. of soya peptone broth, containing 3.32×10^6 units, was filtered, Hyflo being used as a filter aid, without loss in activity. The filtrate was acidified to pH 2.4 by addition of conc. HCl, stirred with 1% (w/v) Hyflo, and filtered by suction through a thin pad of Hyflo. The loss of potency of the filtrate was 1.46×10^6 units, 44% of the original activity. The residue was air-dried overnight, suspended in 3 l. of water, adjusted to pH 8.0 by addition of 40% NaOH, stirred for 30 min., and filtered. The filtrate, having a pH of 7.3, showed an activity of 22 units/ml. It was concentrated *in vacuo* at 30–35°C. to 97 units/ml. This concentrate showed high antifungal activity.

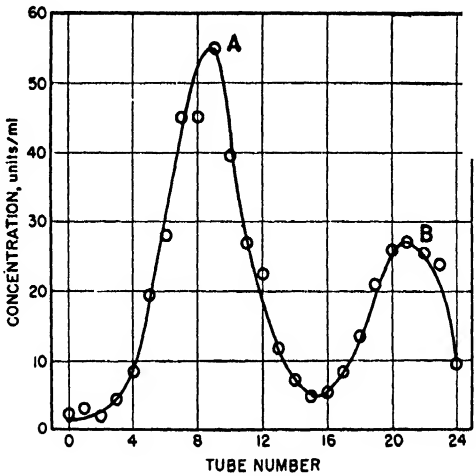

FIG. 3. Distribution curve for a mixture of equal units of neomycin, streptomycin, and streptothricin between borate buffer and pentasol at pH 7.6. A—streptothricin and streptomycin; B—neomycin.

Table V shows that the fungus factor, produced more profusely in the soya peptone medium than in the bacto peptone medium, was not affected by filtration through Hyflo at the pH of the broth after completion of the fermentation. However, it was partly removed from broths by filtration through Hyflo at pH 2.0–2.5, and was completely removed by filtration through 0.5% Darco at that pH.

Factor X was also isolated from 310 ml. of soya peptone broth, having a potency of 69 units/ml., by shaking with 100 ml. of *n*-butanol for 30 min. at pH 2.4. The separated butanol layer was mixed with one-

fourth vol. of water, the pH was adjusted to 7.7, the mixture was shaken 30 min., and the layers were separated. The butanol layer was concentrated *in vacuo* at 30–35°C. to dryness. The residue was picked up in water and lyophilized. A solid, having a potency of 0.5 unit of neomycin/mg. and showing high antifungal activity, was obtained. The water layers from the acid extraction and the alkaline wash showed no antifungal properties. The same result was obtained when the concentrated solution of Factor X, noted above, was subjected to this treatment.

TABLE V

Spectra of Culture Filtrates of Streptomyces 3535

Medium	Treatment of culture filtrates	Dilution units/ml.			
		E. coli	*T. mentagrophytes*	*A. niger*	*P. notatum*
Bacto peptone	Neutral paper filtration	700	20	30	100
Bacto peptone	Neutral Hyflo filtration	700	20	30	100
Bacto peptone	Acid Hyflo filtration	250	0	30	70
Bacto peptone	Acid-Darco filtration	250	0	0	0
Soya peptone	Neutral paper filtration	>1000	30	300	1000
Soya peptone	Neutral Hyflo filtration	>1000	30	100	1000
Soya peptone	Acid Hyflo filtration	>1000	5	50	50
Soya peptone	Acid-Darco filtration	500	0	0	0

Factor X was also adsorbed selectively from culture filtrates at pH 7.9 by 2% (w/v) Norit H 15 or Norit AGNC.

Discussion

The data presented in Fig. 2 definitely establish the fact that neomycin, originally thought to be a single entity, is in reality a mixture of at least 3 different fractions. The first fraction (I), having the lowest distribution coefficient and showing a peak at tube 1, represented about 55% of the total units originally placed in the machine; the second (II), that showing a peak at tube 15, represented about 20% of the original; and the third (III), that having the highest distribution coefficient and showing a peak at tube 48, represented 11% of the original. Hence, it is apparently more correct to discuss the "neomycin complex" in this and related papers than to call the substance obtained by these procedures "neomycin."

Table IV reveals that the 3 fractions obtained above have very similar antibacterial activities, though the third fraction showed the greatest activity against a neomycin-resistant strain of *Mycobacterium* 607(RN), thus indicating that the resistance developed in this organism to neomycin was only against fractions I and II.

As the data in Table V indicate, neomycin was produced in culture filtrates of *Streptomyces* 3535 along with an antifungal agent designated here as "Factor X." Factor X was produced in greater amount in soya peptone medium than in bacto peptone medium. Its production was favored in either medium when less air was supplied to the growing organism.

Neomycin is basic in nature, as shown by its formation of a hydrochloride and a picrate, and by its adsorption on cationic but not on anionic exchangers. It is also adsorbed on most common adsorbents, as shown in Table III. It is readily soluble in water, slightly soluble in methanol, and insoluble in other organic solvents. It can be precipitated from culture filtrates by shaking with 2.5 vol. of 95% ethanol. It is highly stable over the pH range 2.1–9.0, as shown in Table II. Its failure to give a Sakaguchi test shows the absence of a monosubstituted guanidine group, thereby differentiating it from streptomycin.

Neomycin has also been differentiated from streptomycin, and from streptothricin as well, by its distribution pattern in a 24 plate Craig counter-current distribution machine. In the borate buffer system (10) at pH 7.6, neomycin showed a peak at tube 21, streptomycin at tube 9, and streptothricin at tube 8 in their respective distribution curves. The peak at tube 21 in Fig. 3, the distribution curve for a mixture of equal units of neomycin, streptomycin and streptothricin, is characteristic of neomycin, whereas the peak at tube 9 represents streptomycin and streptothricin. Since the peak in the distribution curve for neomycin, in a given system, is different from those given by streptomycin and streptothricin in the same system, neomycin is different from both these antibiotics.

Acknowledgment

The authors wish to express their grateful appreciation to Mr. Edwin G. Bailey and to Mr. Aldrage B. Cooper for valuable technical assistance.

Summary

The production of neomycin in different media has been studied.

This antibiotic was found to be a mixture of at least 3 antibiotics, and it should, therefore, be called the "neomycin complex."

A standard curve for neomycin, different from that for streptomycin, has been obtained, and has been used for the assay of neomycin by the cup method.

The hydrochloride of the "neomycin complex," having an activity of 212 units/mg., has been obtained from culture filtrates of *Streptomyces* 3535 by the following sequence of steps: clarification of broth with charcoal at pH 2.0–2.5, neutralization and adsorption on Decalso, elution with 10% NH_4Cl, adsorption on charcoal, elution with HCl, and chromatography on charcoal. Picric acid has also been used to effect purification of neomycin concentrates.

Neomycin is distinctly different, chemically and biologically, from streptomycin and from streptothricin. The biological differences have been described elsewhere. The chemical differences were illustrated by the distribution pattern that neomycin exhibits in a 24 plate Craig counter-current distribution machine, between various buffers and pentasol.

Neomycin is a basic, relatively stable substance. It is not adsorbed on anionic exchangers.

An antibiotic, having antifungal properties and spoken of as "Factor X," has also been isolated from culture filtrates of *Streptomyces* 3535.

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The Chemical Nature of Urogastrone [1]

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Introduction

Urogastrone is a water-soluble substance found in mammalian urine. Its activity in inhibiting gastric secretion in man and the dog has been summarized (1). With the exception of the work of Gray *et al.* (2), who reported its behavior with respect to precipitants and solvents, and its stability to boiling and peptic digestion, there is very little information available regarding its chemical nature. Our preliminary work showed that the urinary extracts, whether derived from human male or female pregnancy urine, consisted of proteinaceous and polysaccharide-like material in the approximate ratio of 70 and 30%, respectively. Although no reducing substances are detectable without hydrolysis, the preparations, after hydrolysis for 15 min. with 0.6 *N* HCl at 15 lbs. pressure, give substantial amounts of sugar-like material. Inactivation studies were begun with the idea of acquiring more information regarding the nature of the active principle and to determine, if possible, whether or not the anti-secretory substance found in male urine was similar to that found in pregnancy urine. The action of specific group reagents on urogastrone obtained from human male and female pregnancy urine was studied.

Preparation of Urogastrone

The method most commonly used for preparing urogastrone has been the Katzman and Doisy (3) benzoic acid adsorption method for preparing chorionic gonadotropic extracts from human pregnancy urine. Carbon adsorption (4) and $(NH_4)_2SO_4$ (5) precipitation also have been described as useful methods. The extracts used in this study were prepared by the benzoic acid method as follows: Pooled urine samples (preserved with $CHCl_3$) were collected every 24 hr. and the pH was adjusted to 4.5 with acetic acid. Clarification before extraction was accomplished by filtering through

[1] The authors are indebted to Mr. and Mrs. Francis E. Fowler and members of their family, whose generosity has made these studies possible.

a bed of filter aid (Celite 545, Johns-Manville Corp.). The remova of the urogastrone from the urine was effected by the addition of 50 ml./l. of urine of a saturated solution of benzoic acid in acetone. Constant agitation was maintained during the addition of the acetone-benzoic acid solution and was continued for 15 min. afterwards. After standing in the cold overnight, the precipitate was removed from the mixture as a benzoic acid cake. The benzoic acid in the cake was removed by dissolving with an excess of acetone. The acetone-insoluble powder was extracted 3 times with water and a high speed, refrigerated centrifuge then employed to clear the liquor. The active material was precipitated from this aqueous extract by addition of acetone to 80% concentration. The powder was again extracted with water and made up to 20% acetone for precipitating small amounts of inert material. The 20% acetone supernatant was placed in cellophane bags and dialyzed against distilled water for 24 hr. Upon completion of the dialyses the active material was dried by lyophilization or 80% acetone precipitation. The final product from 100 l. of male urine weighs, on the average, 1.3 g.

A 2 mg. dose of the material prepared in the above manner from male urine completely inhibits ulceration in the 150 g. pyloric ligated rat (6). Extracts prepared from pregnancy urine by the same process were of equal or higher potency. Because of the method of preparation, the composition of the anti-ulcer preparation from human pregnancy urine is identical with that of chorionic gonadotropin from the same source. An examination of several commercial chorionic gonadotropic extracts revealed that they were all potent anti-ulcer preparations. Although there was no correlation between the 2 biological tests, further proof was desirable to prove that pregnancy urine urogastrone contains at least 2 factors.

Assay Method

The assay procedure used was that described by Pauls, Wick and MacKay (6). The test substances were administered intraperitoneally as a neutral aqueous solution at the time of pyloric ligation. In order to compare the anti-ulcer activity of test substances it has been found that the comparison may best be carried out by determining the least amount of material required to completely inhibit the ulceration in 80% of the assay animals. In all assays a control group of rats were given saline. In all probability the ulceration in the pyloric ligated rat, as used in these assays, is directly related to gastric secretion, since ulceration may be reduced in extent or entirely prevented by a reduction in gastric juice. The inactivation studies with specific group reagents, which were carried out on male and pregnancy urine urogastrone, were supplemented for comparison purposes in the pregnancy urine preparation with chorionic gonadotropin assays. The chemical reactions were carried out a minimum of 3 times to minimize fortuitous results. After preliminary assays had been carried out for each reaction product to determine the approximate per cent inactivation, at least 20 rats were used for the final anti-ulcer assays and 60 rats were used for the gonadotropin assay at 3 dosage levels. The gonadotropic assays were based on the vaginal cornification in young rats.

Inactivation Procedure

In the following experiments all reaction products were routinely dialyzed before being administered to the test animals.

Acetylation. Ten mg. protein/ml. water; add 0.25 ml. acetic anhydride and let stand overnight; pH of this mixture approximately 3.5.

Ketene. Ketene generated by thermal decomposition of acetone. Pass through a salt-ice trap to condense out unreacted acetone and polymers of ketene. Pass the gas through the protein solution (1 mg./ml. in *M* sodium acetate whose pH is adjusted to 5.9 with glacial acetic acid) for 1.5 hr. The final pH is 4.6.

Benzoylation. Fifteen mg. protein/ml. water. Adjust to pH 9. Add one drop benzoyl chloride to the mechanically stirred protein solution. Stir until the droplet of benzoyl chloride goes into solution, usually about 15 min.; repeat as necessary over a period of 4 hr., maintaining the pH with 0.1 *N* NaOH. A slight precipitate usually starts to form at about the 3rd hour.

p-Diazoniumbenzenesulfonate. Nine mg. protein/ml. water. Adjust pH to either 6.5 or 8.5. Cool in an ice bath and, while mechanically stirring, add 9 mg. of *p*-diazoniumbenzenesulfonate. Stir for 1.5 hr. Quench reaction by dilution. Adjust pH to 7 and dialyze for 24 hr.; lyophilize. The product has an orange color. There always is a considerable increase in weight of lyophilized material (corrected in animal assays).

Nitrous Acid. Ten mg. protein/ml. of 1 *M* $NaNO_2$ in 5% acetic acid at room temperature for 30 min.; pH of solution about 4. Stop reaction with an amount of urea equivalent to $NaNO_2$ used.

Iodine. Ten mg. protein/ml. of 0.005 *M* I_2 in 1 *M* KI and allowed to stand approximately 14 hr. at room temperature—pH approximactly 6.6.

Glutathione. Eleven mg. protein/ml. water. Adjust pH to 9.0 and add 11 mg. glutathione. Dilute to 20 ml. and allow to stand at room temperature under a N_2 atmosphere for 3 days.

p-Chloromercuribenzoic Acid. Fifty-two mg. protein and 52 mg. *p*-chloromercuribenzoate (Na) in 5 ml. NaOH—pH 9. Dilute to 15 ml., adjust pH to 8.7, and allow to react for 1.5 hr.

Hydrogen Peroxide. Seventy mg. protein dissolved in 1 ml. water. Four ml. 30% H_2O_2 added at room temperature and allowed to stand 1 hr. (pH = 6.5).

Glutathione + Benzoyl Chloride. Combine previous procedures.

Glutathione + p-Chloromercuribenzoic Acid. Combine previous procedures.

Discussion

The results of the inactivation studies are summarized in Table I. Gonadotropic potency of pregnancy urine urogastrone is partially or completely destroyed by reagents that destroy or alter the nature of the basic groups, such as free amino groups, guanidino groups of arginine, amidazole groups of histidine, pyrolidine of proline and hydroxyproline, and indole of tryptophan. Acetic anhydride, ketene, benzoyl chloride, *p*-diazoniumbenzenesulfonate and HNO_2, all react with these basic centers and, as indicated in Table I, are capable of destroying gonadotropic activity. With the exception of HNO_2, these reagents can also react with aliphatic hydroxyl groups. Ketene, however, has been reported to be unable to acetylate the free hydroxyls of carbohydrates under the conditions of reaction employed (7).

The anti-ulcer potency of both male and pregnancy urine urogastrone is reduced by more than 50% by acetic anhydride and benzoyl chloride. It is resistant to the action of HNO_2, indicating independence of the anti-ulcer moiety on the basic groups, such as the free amino groupings. The hydroxyl groups appear responsible for the anti-ulcer property. Pregnancy urine urogastrone anti-ulcer activity is partially destroyed by ketene and *p*-diazoniumbenzenesulfonate. Male urogastrone is unaffected by these reagents under the same conditions. It

TABLE I

Comparison of Anti-Ulcer and Chorionic Gonadotropin Inactivation of Pregnancy Urine Urogastrone and Male Urogastrone with Specific Group Reagents

Reagents		Pregnancy urine urogastrone		Urogastrone from male urine
		Gonadotropic inactivation[a]	Anti-ulcer inactivation	Anti-ulcer inactivation
		per cent	*per cent*	*per cent*
Acetic anhydride		95	75	75
Ketene		95	50	0
Benzoyl chloride		100	50	50
p-Diazoniumbenzenesulfonate	pH 6.5	100	0 to 25	0
	pH 8.5	100	50	—
Nitrous acid		90	0	0
Iodine		80	0	0
Glutathione		80	0	0
p-Chloromercuribenzoate		0	0	0
Hydrogen peroxide		95	50	50

[a] For an extensive bibliography and discussion on the reactions of proteins with chemical reagents refer to Herriot, *Advances in Protein Chem.* **3**, 169 (1947); and Olcott, H. S., and Fraenkel-Conrat, H., *Chem. Revs.* **41**, 151 (1947).

must, therefore, be concluded that, although these 2 factors appear to be similar, there must be some fundamental difference involved in the anti-ulcer mechanism of pregnancy urogastrone and male urogastrone.

Iodine, which substitutes *ortho* to the phenolic group of tyrosine and sterically hinders it, destroys gonadotropic activity but leaves the anti-ulcer activity intact. The phenolic nucleus, therefore, seems essential to gonadotropic activity only. The hydroxyl groups involved in the anti-ulcer function are probably aliphatic, and possibly involve the carbohydrate fraction. This seems to be the case with male urogastrone,

as indicated by the ketene experiment. Glutathione destroys only the gonadotropic activity. This indicates the essential nature of the disulfide linkages which this reagent reduces to sulfhydryl.

p-Chloromercuribenzoic acid, which is a specific group reagent for sulfhydryl, failed to destroy either gonadotropic or anti-ulcer potency. Sulfhydryl is, therefore, non-essential.

The H_2O_2 oxidation employed was quite vigorous and would be expected to oxidize amino groups to a variety of compounds up to and beyond the hydroxylamine. Sulfhydryl would also be oxidized, but this group has already been shown to be non-essential. This reagent destroys not only gonadotropic activity, but it also partially inactivates the anti-ulcer factor of both male and female urogastrone. The basic groups (amino, *etc.*) have been shown by the HNO_2 treatment to be non-essential for anti-ulcer activity. Hence, it seems probably that the H_2O_2 oxidation destroys anti-ulcer activity through oxidation of the carbohydrate moiety of the urogastrone.

These studies indicate that urogastrone prepared form pregnancy urine consists of two factors, a gonadotropic factor and an anti-secretory factor, referred to in this paper as the anti-ulcer factor. A similar anti-ulcer factor can be isolated from male urine. In both the male and the pregnancy urine urogastrone, the anti-ulcer function appears dependent upon some mechanism operating through aliphatic hydroxyl groups with evidence pointing, in the case of male urogastrone, to hydroxyl groups of the polysaccharide, which may be chemically bound to the protein.

The importance of the hydroxyl grouping for anti-ulcer protection may be related to the previously reported findings from this laboratory (8) in regard to the position of the hydroxyl group when attached to the benzoic acid molecule. It was found that *o*-hydroxybenzoic acid was an effective anti-ulcer agent, whereas the *m*- and *p*-substituted compounds were relatively inactive. Needless to say, naturally occurring urogastrone appears to contain an aliphatic hydroxyl grouping in contrast to the aromatic ring in salicylic acid.

Summary

Inactivation studies with specific group reagents has indicated that the anti-ulcer activity of urogastrone, whether derived from normal male urine or female pregnancy urine, is dependent on aliphatic hydroxyl groupings. It is suggested that the anti-ulcer factor is associated with a polysaccharide which may be chemically bound to a protein.

Addendum

Since this manuscript has been prepared, McGinty, Wilson and Rodney (9) have shown that bacterial pyrogens in minute quantities are potent anti-ulcer substances. We had observed that 5γ of a pyrogen sample prepared form *Brucellus abortis*, and furnished to us by Dr. Peter Weger (St. Vincents Hospital, Los Angeles), completely inhibits ulceration in our rat assay test. McGinty and coworkers suggest that the anti-ulcer action of urogastrone may be due to its pyrogen content. This possibility concurs with our findings that the anti-ulcer action of urogastrone appears to be associated with a polysaccharide. Evidence against the pyrogen theory is the work of Gray *et al.* (10,11), who prepared pyrogen-free urogastrone. In 1947, Dr. M. H. Kuizenga (The Upjohn Laboratories, Kalamazoo, Mich.) kindly checked for us the pyrogenicity of a sample of urogastrone prepared in this laboratory. The test was carried out in 3 rabbits by injecting into each 5 ml. of a 10 mg./ml. solution. The temperatures of the rabbits were recorded before the injection and at hourly intervals for 3 hr. after the injection. The rise in temperature was 0°, 0.4° and 0°C. It is apparent that no temperature increase was recorded, but it is possible that a temperature lowering effect of the extract may have masked a pyrogen response. Dr. Kuizenga was in agreement with us regarding the anti-secretory activity of this sample.

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A New Colorimetric Procedure for the Determination of Fumaric Acid [1]

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Introduction

Recent studies on the determination of fumaric acid in animal tissues employing chromatography (1) necessitated the development of a comparative method of analysis for the acid. The following procedure was found to be suitable and to be generally applicable to the determination of fumaric acid in biological materials when the amount to be measured is greater than 0.4 mg.

The method, based on the microqualitative test of Steenhauer (3), involves the precipitation of fumaric acid as the copper-pyridyl complex and the subsequent colorimetric determination of the copper thus combined.

Reagents

Ammonium Hydroxide Solution. Eighty-eight ml. of conc. NH_4OH are made to 200 ml. with water. This solution is approximately 10% with respect to ammonia.

Copper-Pyridyl Reagent (conc.). To 20 ml. of 20% copper sulfate are added 8 ml. of pyridine.

Gum Ghatti. A wire screen containing 20 g. of gum ghatti is suspended just below the surface of a liter of water in a glass cylinder. After standing 24 hr., the liquid is strained through a clean cloth.

Pyridine Solution (0.5%). One-half ml. of pyridine is made to 100 ml. with water.

Pyridine Solution (1%). One ml. of pyridine is made to 100 ml. with water.

Sodium Diethyldithiocarbamate (0.2%). Two g. of the reagent are made to volume with water.

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[1] The data in this paper were taken from a dissertation presented by Lawrence M. Marshall in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Wayne University, 1949.

[2] National Institute of Health Junior research Fellow, 1947–48. Present location: Department of Biochemistry, Howard University School of Medicine, Washington 1, D. C.

PROCEDURE

Calibration

To five 15 ml. conical centrifuge tubes is transferred 0.2, 0.4, 0.6, 0.8, and 1.0 ml. quantities, respectively, of a stock solution of fumaric acid having a concentration of 100 mg./100 ml. of solution. Sufficient water is added to each tube so that the total volume is 1 ml.; 0.05 ml. of copper-pyridyl reagent is then added to each tube, and, after shaking, the tubes are allowed to stand until incipient turbidity (usually within 1–2 min.) indicates the formation of the copper-pyridyl fumarate complex. To each centrifuge tube is then added 0.5 ml. of the copper-pyridyl reagent and the tubes are placed in a refrigerator for 1 hr., removed and centrifuged at 200 r.p.m. for 15 min. The supernatant fluid is decanted and the tubes allowed to drain by inverting on filter paper for 3–4 min. The precipitate is washed with 2 ml. of cold (5°C.) 0.5% pyridine solution, and the tubes centrifuged and drained as above. The precipitate is dissolved

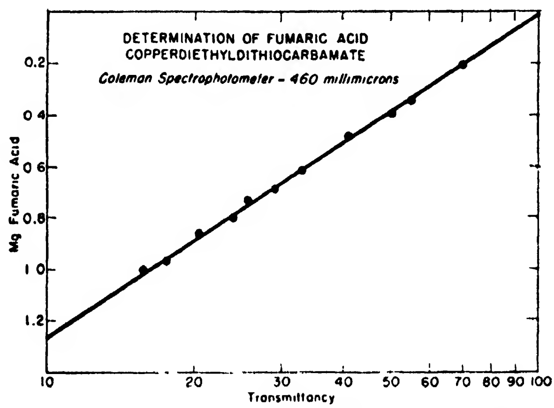

FIG. 1. The calibration curve for the copper-pyridyl method. Transmittancy was measured using the Coleman spectrophotometer at a wavelength of 460 mμ.

in 1 ml. of 20% citric acid and transferred to a 50 ml. volumetric flask, using subsequent 5 and 4 ml. quantities of 20% citric acid solution and 10 ml. of water to insure a quantitative transfer. To each volumetric flask is added 10 ml. of 10% NH_4OH, and the contents made to volume with water. After mixing, a 5 ml. aliquot is placed in a 50 ml. test tube and 1 ml. of gum ghatti and 5 ml. of 0.2% sodium diethyldithiocarbamate are added. The color is allowed to develop for 4 min. and the solutions read in the Coleman spectrophotometer at a wavelength of 460 mμ. The "blank" used in the measurement is composed of citric acid, NH_4OH, gum ghatti and diethyldithiocarbamate in the proportions used for the measurement of the copper solutions. Milligrams of fumaric acid are plotted against transmittancy. The calibration curve employed appears in Fig. 1.

Measurement of Fumaric Acid in Tissue Extracts

A protein-free aqueous extract of minced tissues or of body fluids, or a filtered aqueous medium containing fumaric acid, or a dry chloroform or ether extract dis-

solved in 1% pyridine may be used. A 1 ml. aliquot is transferred to a centrifuge tube and the same procedure is then followed as described under the calibration procedure.

Calculations. The calculation of fumaric acid in the starting material can be given by:

$$\text{Fumaric acid, mg.-\%} = \frac{A \times F \times 100}{W},$$

where:

A = mg. corresponding to transmittancy read;
F = aliquot factor; and
W = original weight of the starting material in g.

Recovery of Added Fumaric Acid

Recovery studies (Table I) involving introduced fumaric acid in urine and egg white showed that the method compared favorably with the chromatographic method (2).

Interference by Other Organic Acids

It has been shown (3) that anisic, anthranilic, benzoic, salicylic, and acetylsalicylic acids, like fumaric acid, will precipitate with the copper pyridyl reagent. Therefore, if significant amounts of these acids are present in the material in which fumaric acid is to be determined, the above procedure must be supplemented to insure their removal.

TABLE I

A Comparison of the Copper-Pyridyl and the Chromatographic Methods for the Analysis of Added Fumaric Acid in Urine and Egg White

Sample	No. of det'n.	Fumaric acid per sample	Copper-Pyridyl method		Chromatographic method	
			Av. fumaric acid found	Standard dev.	Av. fumaric acid found	Standard dev.
		mg.	*mg.*		*mg.*	
Urine	10	3.00	3.14	±0.13	2.98	±0.11
Egg white	10	2.00	2.07	±0.08	1.81	±0.03

This may be accomplished by chromatographing the tissue extract containing fumaric acid on silica gel as previously described (2). Because of their solubility in chloroform, a wash of the silica gel column with 10 ml. of chloroform prior to the elution of the fumarate with 30% amyl alcohol-chloroform completely removes the interfering acids. The data in Table II indicate that this treatment satisfactorily removes these acids. Additional compounds were tested by dissolving 1–5 mg. (depending on the solubility of the compound) in 1 ml. of 1% pyridine and testing for precipitation with the copper-pyridyl reagent under the conditions applied in the analysis. Maleic, succinic, malic, acetic, uric, L-aspartic, L-glutamic, and glycine were not precipitated. With quantities of citric acid greater than 10 g./100 ml. of solution, there was precipitation when the reaction stood over 1 hr. Phenolphthalein, thymol blue, and nicotinic acid showed precipitates.

TABLE II

Analysis for Added Fumaric Acid in Urine in the Presence of Benzoic, Acetylsalicylic and Salicylic Acids

Determination	Fumaric acid			
	Copper-Pyridyl method		Chromatographic method	
	Added	Found	Added	Found
	mg.	*mg.*	*mg.*	*mg.*
1	5.61	5.25	5.61	5.66
2	2.80	2.83	2.80	3.12
3	1.40	1.20	1.40	1.29

The foregoing studies suggest the broad applicability of the copper-pyridyl method for determining fumaric acid in biological materials if the concentration in the sample is 0.4 mg. or more. For smaller concentrations, the chromatographic procedure (2) is more satisfactory.

Summary

A method of analysis for fumaric acid involving the precipitation of fumarate as the copper pyridyl complex, and the subsequent colorimetric determination of the copper, using diethyldithiocarbamate is described. The method has been found satisfactory for determining 0.4 mg. or more of fumaric acid in pure mixtures and in animal tissues and fluids. Other similar organic acids known to occur in animal tissues do not interfere under the conditions described.

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A Yellow Variant of Southern Bean Mosaic Virus

The Isoelectric Points of Yellow and of Regular Southern Bean Mosaic Virus Proteins [1, 2, 3]

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Introduction

A variant of Southern bean mosaic virus (SBMV) was isolated by subinoculation from a bright yellow spot produced in bean plants infected with the original strain of the virus. The variant differs from the parent in that it produces bright yellow rather than yellow-green mottling on leaves of Bountiful beans.

Knight (2) has shown that various strains of tobacco mosaic virus (TMV) differ from each other in amino acid content. Influenza A and influenza B viruses were found by Knight (3) to differ in amino acid content and by Miller, Lauffer and Stanley (7) and Miller (6) to differ in electrophoretic mobility. Since the biological properties of a virus are likely to be determined by its chemical composition, and since the electrokinetic properties are dependent upon the surface chemistry, it seemed possible that a study of the electrophoretic behavior of the yellow variant of SBMV might reveal differences between it and its parent. This paper reports the result of such a study.

Materials and Methods

The Yellow Variant of Southern Bean Mosaic Virus

Examination of a large number of Bountiful bean plants systemically infected with SBMV revealed the occasional presence of a bright yellow spot. Subinoculations were

[1] Condensed from a thesis submitted by Elizabeth MacDonald to the Graduate School in partial fulfillment for the degree, Master of Science.

[2] Aided in part by a grant from The National Foundation for Infantile Paralysis, Inc.

[3] Contribution no. 7-p-49 of the Department of Physics, University of Pittsburgh, Pittsburgh, Pa.

made from such spots, by the rubbing method, to leaves of Kentucky Wonder bean plants, where necrotic local lesions were produced. Subinoculations from the necrotic lesions to Bountiful bean plants resulted in the isolation of a yellow variant.[4]

The variant differs from the original principally in that it produces more intense mottling in Bountiful bean plants. This is shown in Table I. It resembles the parent in type of lesion produced on Kentucky Wonder beans.

Purification of Virus Protein

To obtain material for electrophoretic analysis, virus of the yellow variant was multiplied in Bountiful bean plants. After 3 weeks, the plants were harvested, frozen, and stored in a deep freeze unit. The parent virus, which was a pure strain obtained from The Rockefeller Institute at Princeton, N. J., was also multiplied in Bountiful bean plants and treated in a similar manner. Both virus preparations were purified

TABLE I

Days after inoculation	Strain SBMV	Symptoms (number of plants affected)			
		None	Mild	Moderate	Severe
10	Reg.	0	16	5	1
	Yel.	0	20	3	2
12	Reg.	1	15	5	1
	Yel.	1	7	16	1
15	Reg.	1	11	9	1
	Yel.	2	1	16	6
20	Reg.	2	7	8	5
	Yel.	1	0	11	13

according to a variation of Price's method (9). The thawed plant pulp was first suspended in distilled water. A precipitate resulted, which was removed by centrifugation. The supernatant fluid was then treated with K_2HPO_4, and Price's chemical separation followed thereafter. An additional preparation of the regular strain of SBMV was obtained by centrifugation of the plant juice in a Sharples Supercentrifuge at a rate of 7 cc./min. and a speed of 45,000 r.p.m. It was purified further by differential centrifugation in an angle centrifuge of the Pickels type. A second preparation of the yellow variant was obtained by concentrating the infected plant juice by dialysis against egg albumin according to the method of Lauffer and Price (4), followed by differential centrifugation.

Electrophoresis Experiments

For electrophoresis experiments, virus concentrations of about 4 mg./cc. were dialyzed 30–48 hr. in a buffer of appropriate pH and 0.02 ionic strength. The pH was

[4] The authors wish to express their appreciation to Betty Holt Steranka of the Department of Biological Sciences for her assistance in isolating the yellow strain of SBMV.

measured with a Beckman glass electrode pH meter. Sodium acetate buffers of ionic strength 0.02 were used for pH values below 6.5, and potassium phosphate buffers of the same ionic strength for pH values above 6.5.

Electrophoretic mobilities were determined at 2°C. in a Tiselius apparatus equipped with the Longsworth *schlieren* scanning optical system. Boundaries were permitted to migrate 2 hr. in one direction and an equal period of time in the opposite direction. The average for the ascending and descending boundaries was taken as the mobility at a particular pH value.

Presentation and Discussion of Data

The relationship between pH and mobility of the yellow variant and the original strain of Southern bean mosaic virus is presented in Fig. 1.

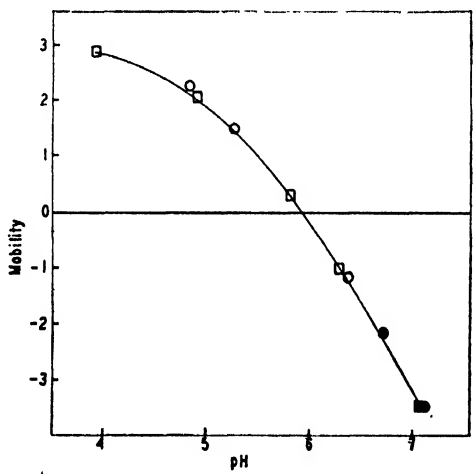

Fig. 1. Electrophoretic mobility, expressed in arbitrary units, of Southern bean mosaic virus protein plotted against pH. ○ Regular strain in sodium acetate buffers, ionic strength .02. ● Regular strain in potassium phosphate buffers, ionic strength .02. □ Variant strain in sodium acetate buffers, ionic strength .02. ■ Variant strain in potassium phosphate buffers, ionic strength .02.

The isoelectric point is shown to be at pH 5.9 for both viruses. Miller and Price (8) had previously reported a value of pH 5.5 for the isoelectric point of the regular strain.

Since the precision of isoelectric points depends primarily upon the accuracy of pH determinations, the pH meter and commerical buffers used in these experiments were standardized against buffers prepared according to standard methods (1,5). The greatest difference found

between the reference pH and that obtained when the Beckman pH meter was standardized with commercial buffers was 0.02 pH unit.

The data of Fig. 1 show that, in contrast with the results obtained with influenza A and influenza B virus preparations, the mobilities and isoelectric points of the regular and variant Southern bean mosaic virus protein preparations are indistinguishable. However, the isoelectric point of 5.9 is not in agreement with the value of 5.5, obtained by Miller and Price (8) at Princeton, N. J. In both the experiments carried out there and in this laboratory, buffers of ionic strength 0.02 were used. In addition, sodium acetate buffers were used for the pH values immediately adjacent, on both sides, to the two different isoelectric points. In obtaining these isoelectric points, great care was exercised in each laboratory in the use of well standardized and accurate pH meters.[5] In view of these facts, it seems unlikely that the difference is due to experimental error. It is possible that the reason for the difference in the isoelectric point obtained by these two laboratories on the regular strain of Southern bean mosaic virus is that, in transferring it from The Rockefeller Institute to different environmental conditions at Pittsburgh, a spontaneous mutation occurred.

Summary

1. A variant which characteristically produces yellow mottling on Bountiful bean plants was isolated from a yellow spot on Bountiful bean plants systemically infected with regular Southern bean mosaic virus.

2. The isoelectric points of the proteins of the yellow and of the regular Southern bean mosaic virus were found to be at pH 5.9, in contrast with the value of 5.5 previously found for the regular virus protein.

3. The electrophoretic mobilities of the two proteins were identical over the range from pH 3.9 to pH 7.2.

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[5] Dr. G. L. Miller has kindly examined preparations of both our regular and variant strains of virus and obtained an isoelectric point in reasonable agreement with that indicated in Fig. 1.

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The Interaction of Tobacco Mosaic Virus and of Its Degradation Products with Dyes

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When tobacco mosaic virus is denatured by heating, it splits into two components, a nucleic acid and an insoluble protein. As will be shown, the intact virus exhibits entirely different staining properties from those of the insoluble protein and of the nucleic acid. The staining properties of the nucleic acid depend very much on its state of degradation. A comparison is also made with the staining properties of serum and of egg albumins.

Staining of the Intact Virus

At pH values above its isoelectric point [pH 3.2–3.5 in buffers (1,2) and pH 3.9 in water (3)], tobacco mosaic virus is negatively charged and, therefore, should combine with basic dyestuffs by virtue of Coulombic attraction.

A study was made of the interaction of the basic dye acriflavine (trypaflavin) with the virus at a pH value above the isoelectric point of the virus.

The tobacco mosaic virus was purified by differential centrifugation by the method of Stanley (4), and the purified virus was then resuspended twice in water. The aqueous solution of the virus had a pH value of about 6.5 and an electrical conductivity corresponding to a NaCl solution of about 10^{-4} *M*. The binding of the dye to the virus was determined by sedimenting the virus from a solution containing the dye. The amount of dye bound by the virus is taken as the difference between the amount of dye originally present and the amount in the supernatant fluid after centrifugation (free dye). Fourteen Lusteroid centrifuge tubes containing 5 ml. of virus solution

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(2.55 mg. protein/ml.) and 5 ml. of dye solution were centrifuged for 1 hr. at 30,000 r.p.m. in a Bauer-Pickels air-driven centrifuge. The virus-free supernatant fluid was removed and the dye concentration was determined by measuring the optical density at $\lambda = 446$ mμ, the wave length of maximum light absorption of the dye, in a Beckman spectrophotometer and compared with an optical density-concentration calibration curve for the dye. It was found that the centrifuge tubes were not stained by the dye nor did any dye sediment in a virus-free solution. The experiments of absorption of the dye by the virus were made in water, 0.01 *M* NaCl and 0.10 *M* NaCl. In Fig. 1 are illustrated the experimental results; the moles dye bound per unit weight of virus *vs.* concentration of free dye. In water, the dye bound follows a Langmuir isotherm (5). Such a curve is expected on purely statistical grounds if it is asusmed that the molecules adsorbed do not interact with one another (6) and has been observed for the methyl orange-serum albumin system by Klotz and his coworkers (7). Klotz presents his data as the reciprocal of the quantities given in Fig. 1 [see also Klotz (8)].

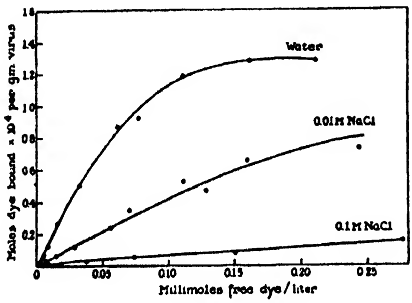

FIG. 1. The binding of acriflavine by tobacco mosaic virus.

The amount of dye bound/g. of virus is equal to $\alpha c/(K + c)$, where c is the molar concentration of free dye, α is the limiting value of the binding for high dye concentrations, and K is the dissociation constant of the dye ion and the charged group on the protein with which it binds. From the curve for water of Fig. 1 it is seen that the limiting value of binding, α, is equal to 1.3×10^{-4} *M* of dye/g. of virus. This value is identical with that obtained for the number of carboxyl groups of the virus in water at pH 6.5 as obtained by acid-base titrations (3). Taking the molecular weight of the virus as 40,000,000, then the maximum number of dye molecules adsorbed on a single virus particle is 5.2×10^3. For low dye concentrations the amount of dye bound/g. of virus in water is given by $\alpha\, c/K$ and from Fig. 1, K is calculated to be equal to $10^{-4.1}$. The dissociation constant K is considerably increased in the presence of salts.

Methyl orange and rose bengal, both negatively charged ions, are not bound to the virus under the same experimental conditions as those performed with acriflavine.

Color Shift of Dyes with Virus Nucleic Acid

When a solution of the purified tobacco mosaic virus containing a small amount of salt is heated above 90°C., the virus breaks down into essentially two components—nucleic acid and an insoluble protein containing no phosphorus or carbohydrate (9,10). The nucleic acid has been shown by Loring (11), by a chemical analysis of the carbohydrates and the purine and pyrimidine bases, to be of the ribose type.

In the present work the virus nucleic acid was obtained and purified by the method of Cohen and Stanley (10), and the insoluble protein was washed several times with 0.1 *M* NaCl, and then with water, and was saved for experiments to be described in the next section of this paper. These nucleic acid preparations had an intrinsic viscosity of 13.

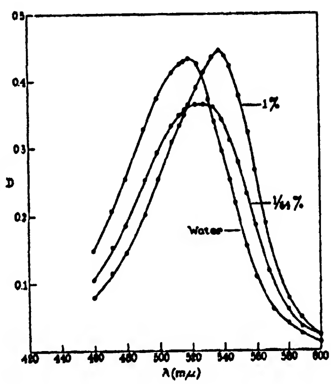

Fig. 2. Absorption spectra of safranin (1.41×10^{-5} *M*) in the presence of varying concentrations of nucleic acid from tobacco mosaic virus.

In the presence of the virus nucleic acid, at pH 7.0, several dyes, including toluidine blue, methylene blue, and safranin, showed an alteration in the color of the dye from that which it possesses in water alone. In Fig. 2 is illustrated the absorption spectra (absorption given in terms of the optical density, *D*) of safranin in the presence of varying amounts of virus nucleic acid. The color shift, which can easily be observed by eye, varies from a maximum of 520 mμ (orange colored) for no nucleic acid present to a maximum of 540 mμ (red colored) for large amounts of nucleic acid present. For low nucleic acid concentrations, the spectrum is a composite of the altered spectrum of the dye bound plus the spectrum of the free dye in solution. The results of Fig. 2 are identical with those found by Michaelis (12) for phenosafranin in the presence of yeast nucleic acid.

The interaction of the dye with nucleic acid is influenced by high salt concentrations. Thus, if NaCl is added to the safranin-nucleic acid mixture in excess of 0.4 *M*, the color shift due to the nucleic acid is somewhat depressed. This may be due to a competition between sodium ions, which are present in large excess, and the basic dye cation for binding with the phosphate groups of the nucleic acid, or it may be due to incipient salting-out of the dye (12). However, since the salt concentration dealt with in this paper is equal to 0.1 *M*, where this salt effect is not detectable, we shall not consider this factor further.

By far the most important factor influencing the dye-nucleic acid interaction is the state of degradation of the nucleic acid. Thus, if the solution of safranin and the virus nucleic acid is kept at pH 11.9, where

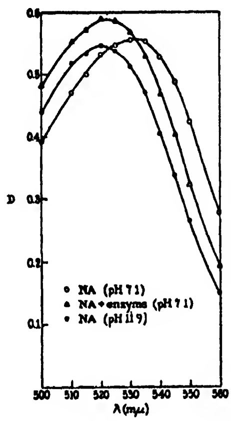

FIG. 3. Absorption spectra of safranin (1.69×10^{-5} *M*) in the presence of nucleic acid (0.035%) from tobacco mosaic virus with and without crystalline ribonuclease (2.1 γ/ml.) at pH 7.0 and the nucleic acid at pH 11.9.

the nucleic acid is degraded, the solution exhibits the color which the dye has at this pH when no nucleic acid is present (Fig. 3). The virus nucleic acid is apparently also degraded by ribonuclease, since this nucleic acid at pH 7 to which crystalline ribonuclease[3] was added, failed to shift the color of safranin in water (Fig. 3). Yeast nucleic acid showed a similar behavior in the presence of ribonuclease. Kunitz (13) has shown that yeast nucleic acid is broken down by the action of ribonuclease into fragments one-eighth the size of the molecule and a similar result might be expected for the virus nucleic acid.

The color shift of safranin is also suppressed when thymus nucleic acid is subjected to analogous treatment. Thus, thymus nucleic acid

[3] We are indebted to Dr. M. Kunitz for providing us with this enzyme and with the desoxyribonuclease described below.

causes the spectra of safranin to shift from 520 mμ to 540 mμ, but, if the solution is brought to pH 11.9, the color returns to that for the dye in water alone. A similar suppression of the shift is obtained by adding desoxyribonuclease to the thymus nucleic acid. A 0.02% solution of thymus nucleic acid containing 0.0025 M $MgCl_2$ and a small amount of gelatin was made. As McCarty (14) has shown, $MgCl_2$ is necessary for desoxyribonuclease activity and gelatin aids in stabilization of the protein. The solution shows with 1.69×10^{-5} M safranin a maximum at 540 mμ typical of nucleic acid (regardless of source). To this solution was added 50 γ of crystalline dexosyribonuclease, which has recently been crystallized by Kunitz (15), and the dye was added after 15 min. digestion. The maximum in the spectra of the dye moved down to 524

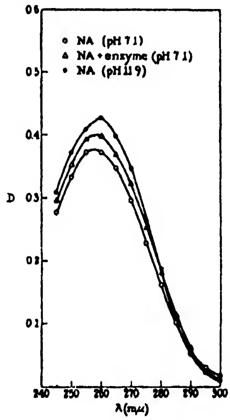

FIG. 4. Ultraviolet absorption spectra of nucleic acid (0.0022%) from tobacco mosaic virus with and without crystalline ribonuclease (2.1 γ/ml.) at pH 7.0 and the nucleic acid at pH 11.9.

mμ, *i.e.*, toward its value in water. Similar experiments were performed with yeast nucleic acid and the virus nucleic acid, and also using ribonuclease under the same conditions given above. It was found that ribonuclease had no effect on thymus nucleic acid but does act on yeast nucleic acid and on the virus nucleic acid. It was also found that desoxyribonuclease has no effect on the yeast nucleic acid or on the virus nucleic acid.

Kunitz (16) has shown that the ultraviolet absorption at 260 mμ of yeast nucleic acid is increased when the nucleic acid is digested with ribonuclease. We have also found this result with virus nucleic acid (Fig. 4). A similar result is obtained by raising the pH of the virus nucleic acid solution to 11.9 (Fig. 4) and, therefore, it may be reasoned

that the effect of the latter treatment involves a similar depolymerization process as that found by enzyme digestion (13).[4]

Since all the treatments of nucleic acid described above involve a breakdown into smaller molecules, it appears, as Michaelis (12) has suggested [see also Michaelis and Granick (17)], that the color shift of the dye in the presence of nucleic acid takes place when the dye cation combines with the highly charged phosphate groups which are arranged along the nucleic acid molecule. When the nucleic acid molecule is degraded, however, the dye behaves as though no nucleic acid is present.

Experiments similar to those described earlier for acriflavine were also carried out with safranin and the intact virus. As with acriflavine, safranin is bound to the virus and the amount of binding decreases with the addition of NaCl. In the presence of freshly purified intact virus, no shift in color is observed for safranin although the dye is bound to the virus particles. Thus, a 2% solution of the virus with 1.69×10^{-5} *M* safranin showed, when the optical density was corrected for the light scattered by the virus, the same absorption spectra as that of safranin in water alone. When the virus solution is heated and safranin added, however, a color shift becomes immediately evident. No color shift is observed when the virus is allowed to stand at room temperature for 3 days at pH 11.9, although nucleic acid is released by this treatment, but it is in the depolymerized state as discussed above.

The failure of the intact virus to show the typical nucleic acid staining reaction indicates that the phosphate groups of the nucleic acid in the intact virus are not available for chemical reactions. This finding is in agreement with Loring (18) that ribonuclease has no enzymatic activity for the intact virus.

Staining of the Insoluble Heat-Denatured Product

The insoluble protein residue obtained by the method described in the previous section was shaken up in 0.1 *M* sodium phosphate buffer at pH 7.0 and used in this form. Since the insoluble protein is easily sedimented by low speed centrifugation, the samples shaken up with the dye under investigation were sedimented in a clinical centrifuge at 1500 r.p.m. for 15 min. The concentration of the dye in the clear supernatant was determined colorimetrically in the Beckman spectrophotometer at a wave length corresponding to the maximum in the absorption spectra of the dye. The concentration of the protein was

[4] Recently one of us (G.O.) has found that there is a marked decrease in the infrared absorption peak at 1230 cm.$^{-1}$ on depolymerization of nucleic acid. This appears to be due to loss of the phosphoric ester linkage. The details will be published elsewhere.

determined by measuring the nitrogen content of sediments of the protein suspensions containing no dye.

It was found that all the dyes investigated are bound in great quantities to the insoluble protein. In Fig. 5 is shown the amount of binding as a function of free dye concentration. As can be seen, the amount of acriflavine bound to the insoluble protein greatly exceeds that bound to the intact virus under the same conditions. Furthermore, the acid dyes, methyl orange and rose bengal, are also strongly bound to the insoluble proteins but are not bound to the intact virus. Rose bengal is so completely bound that the data cannot be represented on the same scale as that of Fig. 5. For example, it was found that a suspension of 0.02 g. of protein/ml. with 7×10^{-4} M rose bengal, when centrifuged, showed no dye in the clear supernatent. Dialysis experi-

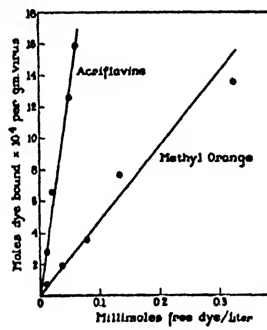

FIG. 5. The binding of methyl orange and acriflavine to the insoluble protein obtained from tobacco mosaic virus by heat denaturation.

ments using sausage casings under the same conditions of salt, protein and dye gave the same results. Unlike the results of binding of acriflavine in water to intact virus, the curves of Fig. 5 do not show any saturation value of binding. This suggests that the binding of the dye to the insoluble protein does not take place on specific amino acid residues, since there is not enough of one kind of amino acid residue in tobacco mosaic virus [see the amino acid analysis of Knight (19)] to account for such great binding. Probably no multilayer absorption of the dye or sticking of dye in the form of micelles is taking place since then theory shows (see, for example, Brunauer (20), Chapter 6) that the curve of the amount of dye bound *vs.* free dye would curve upwards. It is interesting to note that methyl orange and rose bengal are negatively charged, yet bind strongly to the insoluble protein which carries a net negative charge. The electrophoretic mobility of the denatured

protein in 0.1 *M* phosphate buffer at pH 7.0 as measured in a Kunitz-Northrop microelectrophoresis apparatus was found to be the same as that for collodion particles coated with the intact virus. It was further found that the isoelectric point of the insoluble protein in 0.02 Sorenson's citrate-HCl buffers is pH 3.45 ± 0.05, a value which is identical with that measured for the intact virus in these buffers (3).

The fact that the denatured protein is insoluble but possesses the same net charge as that of the intact virus suggests that the denaturation consists of a presentation, probably from the interior of the molecule, of hydrophobic side chain groups, which can bind with dyes by van der Waals forces. Our picture of the denaturation of the protein is contrary to that suggested by Mirsky and Pauling (21) and is closer to that suggested by Abramson, Moyer and Gorin [(22), p. 90]. Rose bengal, which contains much more highly polarizable groups, such as iodine and chlorine-substituted benzene rings, than do the other dyes studied, and which binds more completely than do the other dyes examined, indicates that van der Waals force is the predominating type of force responsible for this binding.

Egg albimin shows a similar reaction with rose bengal to that shown by tobacco mosaic virus. Thus, it was found from dialysis experiments that rose bengal is only slightly bound to native egg albumin in 0.1 *M* phosphate buffer at pH 7.0. If, however, egg albumin is denatured (becomes a turbid suspension at pH 7.0) by heating to 100°C., the dye is bound in large quantities. Incidentally, the isoelectric point of the denatured egg albumin as determined by microelectrophoresis is the same as that for the native protein.

Bovine serum albumin, on the other hand, exhibits an entirely different behavior. We found by dialysis experiments that rose bengal is adsorbed in large quantities to *native* serum albumin. This is consistent with the results of Klotz and his coworkers (7,8,23,24) who found that the acid dye, methyl orange, is bound to native serum albumin at pH 6.8. He and Walker (24) suggest that a necessary condition for the binding of methyl orange to serum albumin is the presence of the positively charged ϵ amino group of lysine, since binding is decreased in the region of pH 10 where the ϵ amino acid residue of lysine becomes electrically neutral. It is not clear, however, why other proteins, such as tobacco mosaic virus and egg albumin, which contain lysine, do not adsorb the dye in the native state. The unusual behavior of serum albumin is also manifested by its ability to combine, apparently by

van der Waals forces, with fatty acids (25,26,27) and other substances (28,29) and by its unique physiological role in the blood stream (30).

Summary

Acriflavine, a basic dye, binds to purified tobacco mosaic virus according to a Langmuir isotherm. The dissociation constant for the dye-virus complex is equal to $10^{-4.1}$ and a maximum of 1.3×10^{-4} moles of dye are bound/g. of virus.

The intact virus does not show the typical nucleic acid staining reaction (color shift with safranin and other metachromatic dyes). On heating the virus, however, the solution shows the nucleic acid staining reaction. The color shift does not take place when the nucleic acid is degraded by ribonuclease or by alkali. Changes in the ultraviolet spectrum of the nucleic acid are also observed on degradation.

The insoluble protein obtained by heating the virus binds strongly with several dyes. Egg albumin has some staining properties similar to those of the virus but serum albumin exhibits a different behavior.

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The Mechanism of Resistance to Sulfonamides. IV. A Comparative Study of the Amino Acid Metabolism of *Staphylococcus aureus* in Relation to the Mechanism of Resistance[1]

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Introduction

As one mechanism of resistance to sulfonamides, the idea had been suggested that, in the presence of the drug, bacteria produce an increased amount of *p*-aminobenzoic acid as antagonist to the drug (1). However, the results obtained by Sevag and Green (2) with *Staphylococcus aureus*,[2] Yegian *et al.* (3) with *Mycobacterium ranae*, and those obtained by Lemberg *et al.* (4) with *Escherichia coli* are not compatible with this idea. The latter investigators isolated and characterized both *o*- and *p*-aminobenzoic acid and found that the resistant cells did not produce more *p*-aminobenzoic acid than the parent susceptible strain,

[1] This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and also by a grant from the Josiah Macy, Jr., Foundation.

[2] Sevag and Green (2) stated that the large amount of the diazotizable amine accumulated in the culture of fluids of *Staphylococcus aureus* is not *p*-aminobenzoic acid. They suggested that it is derived from tryptophan and may consist of a mixture of *o*-aminobenzoic acid and kynurenine. In a personal communication Drs. W. Shive and E. Beerstecher, Jr., University of Texas, Austin, Texas, have kindly informed us that the accumulated amine in the culture fluid of *Staphylococcus aureus* is kynurenine. We quote from Dr. Shive's letter, "This identification was made on the basis of properties of a concentrate of the amine, which, on diazotization, condensed with an amine to form a dye at the same rate as kynurenine. Also, on paper chromatographs in several different solvents, the amine from *Staphylococcus aureus* had R_f values identical with those of kynurenine. These and other properties allowed identification without resorting to the usual chemical identifications."

and the amount was insufficient to account for the resistance to sulfathiazole. These observations would then contradict the idea that these resistant cells produce an increased amount of *p*-aminobenzoic acid. Despite the above-cited quantitative chemical determinations, the idea still persists that resistant cells produce more *p*-aminobenzoic acid as a mechanism of resistance to sulfonamides (5,6). The data presented here show that the drug-resistant cells are deficient in ability to synthesize various amino acids.

It had previously been found that the resistance of *Staphylococcus aureus* to sulfonamides involves a modified amino acid and glucose metabolism (2). In this study, an attempt has been made to determine how these degradative mutations associated with resistance have affected the utilization and synthesis of amino acids by *Staphylococcus aureus* resistant to sulfonamides.

Experimental

Source of the Strains of Staphylococcus aureus

Two pairs of related strains of *S. aureus*, 1A and 1E and 3A and 3E, sulfonamide-susceptible and sulfonamide-resistant, were used. The strain 1A was the susceptible strain used by Sevag and Green (2). The drug-susceptible strain 3A was isolated from a clinical case. Sulfathiazole-resistant strains 1E and 3E were rendered resistant to sulfathiazole by exposing the susceptible strains 1A and 3A every 2–3 days to increasing concentrations of sulfathiazole in casein hydrolyzate medium over a period of 8 months. The training of the resistant strains was finally completed in a concentration of 168 mg.-% sulfathiazole. The parent susceptible strains were cultured concurrently in casein hydrolyzate medium without the addition of sulfathiazole. The resistant strains were found to be at least 20-fold more resistant to the drug than the parent susceptible strains.

Growth Medium

The composition of the basal synthetic amino acid medium is outlined in Table I. Various modifications of this basal medium were used with and without 0.5% glucose.

Concentration of Sulfathiazole

The concentration of sulfathiazole used in the various media was 0.5 mg.-% (5 γ/ml.) for the susceptible organism in the absence, and 10 mg.-% (100 γ/ml.) in the presence of glucose. For the resistant organism it was 10 mg.-% in the absence, and 100 mg.-% (1000 γ/ml.) in the presence of glucose.

Inoculum

The stock cultures consisted of cells grown on extract agar slants for 48 hr. at 37°C. and then stored at 6°C. Cells harvested from the second successive 18 hr. transfer on

TABLE I

Synthetic Amino Acid Growth Medium[a]

Ingredient	mg./1000 ml.
Thiamine chloride	1
Nicotinamide	1
$MgSO_4 \cdot 7H_2O$	12.35
$FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$	8.1
L-Cysteine hydrochloride	200
DL-Alanine	900
DL-Valine	365
DL-Leucine	667
Glycine	375
L-Proline	525
DL-Aspartic acid	313
DL-Isoleucine	490
DL-Methionine	525
L-Glutamic acid	675
DL-Phenylalanine	600
L-Tyrosine	30
L-Arginine monohydrochloride	375
L-Histidine	375
DL-Lysine	375
L-Tryptophan	20

[a] The stated amounts of amino acids, vitamins, and salts were dissolved in sufficient $M/30$ phosphate buffer (pH 7.35) to produce a final volume of 1 l.

extract agar were washed with and suspended in $M/30$ phosphate buffer (pH 7.35). The final concentration of the cell suspension in phosphate buffer was so adjusted that 0.05 ml. of the cell suspension resulted in an inoculum of approximately 250,000 viable organisms/ml. of medium.

Measurement of Cell Growth

Growth was measured as turbidity determined in the Klett-Summerson photoelectric colorimeter (filter 560 mμ). The weight of the organisms was obtained by reference to a turbidity curve based on dry weights. The turbidity-dry-weight curve was obtained in the following manner: the cells of *S. aureus* resulting from growth in casein hydrolyzate medium were harvested and washed twice with water. A stock suspension was diluted to volumes of 10 ml. each in standard Klett-Summerson photoelectric tubes with $M/30$ phosphate buffer (pH 7.35) and the turbidity of each was read in the Klett-Summerson photoelectric colorimeter using filter No. 56. Aliquots of the stock suspension similar to those used for turbidity measurements were dried to constant weight at 106°C. The dry weights obtained ranged from 0.92 to 13.8 mg. A curve was then constructed from the turbidities and the corresponding dry weights. This curve was not a straight line function. Based upon over 100 determinations, the suspensions of washed susceptible and resistant cells harvested from

various growth systems having the same turbidity per unit volume of suspension contained, within 10%, the same total solid content as determined by bringing them to constant weight at 106°C.

Glucose

Glucose was determined by the Folin-Wu method (7).

Results [3]

Comparison of the Rate of Growth of Staphylococcus aureus Susceptible and Resistant to Sulfathiazole in the Presence and Absence of Glucose or Pyruvate

The growths of the resistant strains 1E and 3E in the absence of glucose and pyruvate lag behind those of the related susceptible strains 1A and 3A for at least 72 hr. (Table II). Glucose shortens this lag period to 48 hr. for the resistant strain 3E, but, in the case of the resistant strain 1E, glucose does not enable this strain to produce a growth equal to that of the related susceptible strain 1A even at 72 hr. In the presence of pyruvate, though the growth of the resistant strain 1E is evident at an earlier period (48 hr.) than in its absence, nevertheless, this growth in the presence of pyruvate does not equal that of the susceptible strain 1A as late as 120 hr. The growth of the resistant

TABLE II

Growth of Strains of Staphylococcus aureus Susceptible and Resistant to Sulfathiazole in Synthetic Medium[a]

Strain \ Hours	Mg. of cells/10 ml. medium								
	No glucose			Glucose		Pyruvate			
	48	72	120	48	72	48	72	96	120
1A—Susceptible	1.1	2.4	2.4	7.0	7.0	3.6	3.9	—	—
1E—Resistant	0	tr	2.1	3.2	3.9	0.7	1.1	—	1.8
3A—Susceptible	0.2	0.5	0.9	6.5	6.5	0.9	2.1	2.6	3.2
3E—Resistant	0	0.2	1.8	4.8	6.3	0	1.3	3.9	4.9

[a] The composition of the synthetic medium is given in Table I.

[3] The results of the single experiments presented in this paper are in each case representative of a group of experiments, the results of which were qualitatively similar.

TABLE III

The Effect of Glucose and Sulfathiazole on the Amino Acid Requirements of Staphylococcus aureus 1A (Susceptible) and 1E (Resistant)

Amino acid omitted from synthetic medium	Mg. of cells/10 ml. medium										Growth ratio Strain 1A/Strain 1E	
	1A-Susceptible					1E-Resistant						
	No glucose[a]			0.5% glucose[b]		No glucose[a]			0.5% glucose[b]			
	Control	ST 0.5 mg.-%	ST 10 mg.-%	Control	ST 10 mg.-%	Control	ST 10 mg.-%	ST 100 mg.-%	Control	ST 100 mg.-%	No glucose	0.5% glucose
1. None	2.4	2.6	0	7.0	0	2.1	2.6	1.8	3.9	4.5	1.11	1.79
2. Alanine	1.6	1.6	0	7.1	0.6	2.4	1.5	1.3	3.2	4.8	0.66	2.22
3. Leucine isoleucine	0	0	0	6.9	0	0	0	0	6.1	4.9	0	1.13
4. Glycine	0.8	1.1	0	0.7	0	0	0	0	0.8	0.8	∞	0.99
5. Proline	1.7	2.4	0	0	0	0.7	0.2	0.2	0	0	2.27	0
6. Aspartic	2.6	2.1	0	5.6	0	2.5	2.4	1.2	4.3	4.5	1.05	1.31
7. Glutamic	1.1	0	0	7.0	0	0.4	0.3	0	4.4	2.2	2.87	1.60
8. Methionine	1.0	0.6	0	4.3	0	0.4	0.4	0.2	3.9	2.7	2.73	1.08
9. Phenylalanine	1.1	1.5	0	4.7	0	1.7	0.5	0	2.7	3.4	0.62	1.73
10. Tyrosine	1.4	1.7	0	5.4	0.4	2.1	2.0	1.3	3.4	4.5	0.65	1.62
11. Histidine	1.9	1.6	0	5.5	0	2.4	1.2	1.2	3.8	0	0.77	1.44
12. Lysine	2.5	1.1	0	5.6	0	1.0	0.5	0.4	3.5	5.8	2.60	1.59
13. Tryptophan	1.8	1.5	0	6.7	0.6	1.9	0	0	3.2	0	0.96	2.08

[a] Growth period—120 hr.
[b] Growth period—72 hr.
No growth was observed when valine, leucine, or arginine were omitted.

strain 3E lags for 48 hr. behind that of the susceptible strain 3A, but exceeds it at 96 hr.

These results, in general, then would indicate that resistance is correlated with a slower rate of growth.

Utilization of Preformed Amino Acids in the Presence and Absence of Glucose by Staphylococcus aureus, Strains 1A and 1E

The *ratios* of growth of strain 1A to that of strain 1E in the absence and presence of glucose are 1.11 and 1.79, respectively (Table III, last column). This would indicate that, in the absence of glucose, the utilization of amino acids by the resistant and the susceptible strains for growth is of similar efficiency. However, in the presence of glucose, the growth *ratio* is definitely in favor of the susceptible strain 1A. It can also be seen that the amount of glucose consumed by each 100 mg.

TABLE IIIA

The Effect of Sulfathiazole on the Relation of Glucose Utilization to Amino Acid Requirements of Staphylococcus aureus

Amino acid omitted from synthetic medium[a]	1A Susceptible: mg. of glucose consumed/100 mg. cells		1A Susceptible: Glucose ratio	1E Resistant: mg. of glucose consumed/100 mg. cells		1E Resistant: Ratio of glucose utilization	Ratio of glucose utilization Strain 1A / Strain 1E
	Control	ST 10 mg.-%	Control / ST	Control	ST 100 mg.-%	Control / ST	
1. None	698	0	∞	1253	1119	1.11	0.56
2. Alanine	658	470	1.40	1180	1006	1.17	0.56
3. Leucine iso-leucine	698	0	∞	796	1042	0.77	0.88
4. Glycine	1300	0	∞	1860	1883	0.99	0.70
5. Aspartic	859	0	∞	1125	1137	0.99	0.76
6. Glutamic	696	0	∞	1120	2126	0.53	0.62
7. Methionine	1103	0	∞	1200	1363	0.88	0.92
8. Phenylala-nine	1014	0	∞	1765	1516	1.16	0.57
9. Tyrosine	856	833	1.02	1397	1150	1.22	0.61
10. Histidine	853	0	∞	1232	0	∞	0.69
11. Lysine	873	0	∞	1390	922	1.51	0.63
12. Tryptophan	730	860	0.85	1512	0	∞	0.48

[a] No growth was observed when valine, leucine, proline, or arginine were omitted.

of the susceptible cells is only 56% of that amount of glucose consumed by the same weight of resistant cells under identical conditions (Table IIIA). It would thus appear that the resistant cells in contrast to the susceptible cells have undergone, during the development of resistance to sulfathiazole, a decrease in efficiency in coordinating amino acid and glucose metabolism related to growth in the absence of sufathiazole.

TABLE IV

The Effect of Glucose and Sulfathiazole on the Amino Acid Requirements of Staphylococcus aureus 3A (Susceptible) and 3E (Resistant)

Amino acid omitted from synthetic medium	mg. of cells/10 ml.								Growth ratio Strain 3A / Strain 3E	
	3A Susceptible				3E Resistant					
	No glucose[a]		0.5% glucose[b]		No glucose[a]		0.5% glucose[b]			
	Control	ST 0.5 mg.-%	Control	ST 10 mg.-%	Control	ST 10 mg.-%	Control	ST 100 mg.-%	No glucose	0.5% glucose
1. None	0.9	1.0	6.5	0.7	1.8	0.6	6.3	5.3	0.51	1.03
2. Alanine	0	0	6.5	0	0	0	6.4	7.2	0	1.01
3. Glycine	0.7	0.7	1.0	0.6	0.4	0	1.6	1.9	1.77	0.63
4. Proline	1.1	0.8	0.3	0	0.2	0	0.2	0	6.47	1.26
5. Aspartic	1.1	1.0	2.9	0	0	0	0	0	∞	∞
6. Glutamic	0.6	0	3.8	0	0	0	5.5	0.6	∞	0.68
7. Methionine	0.7	0.5	4.3	0	0	0	6.9	0	∞	0.62
8. Phenylalanine	0.7	0.6	4.0	0.4	0.2	0	8.0	3.5	4.41	0.50
9. Tyrosine	0.8	0.8	4.4	0	0.2	0	7.4	5.6	4.42	0.59
10. Arginine	0.6	0.4	0.4	0.3	0.2	0	0.4	0.2	3.59	1.00
11. Histidine	0.8	0.9	5.9	0	0	0	6.4	0	∞	0.91
12. Lysine	0.9	0.8	4.3	2.0	0	0	6.1	4.8	∞	0.70
13. Tryptophan	0.9	0.8	3.9	0	0.3	0.2	5.6	0	2.84	0.69

[a] Growth period = 120 hour.
[b] Growth period = 72 hr.
In absence of valine and leucine, no growth occurs.

Strains 3A and 3E. The *ratios* of growth of strain 3A to that of strain 3E in the absence and presence of glucose are, respectively, 0.51 and 1.03 (Table IV, last column). This would indicate that the resistant cells are more efficient in utilizing amino acids for growth than the susceptible cells. The inefficiency of the susceptible strain in this respect is compensated for by the metabolism of glucose.

The Effect of Sulfathiazole on the Utilization of Preformed Amino Acids by Staphylococcus aureus

Strains 1A and 1E. The growth of the susceptible strain 1A, in the absence of glucose, is not inhibited by 0.5 mg.-% sulfathiazole (Table III); increasing the concentration of the drug to 10 mg.-% inhibits completely the growth of this strain in the absence and presence of glucose. In contrast, the resistant strain 1E, in the absence of glucose, shows 20% increase in growth in the presence of 10 mg.-% sulfathiazole and only 17% inhibition of growth by 100 mg.-% sulfathiazole.

TABLE IVA

The Effect of Sulfathiazole on the Relation of Glucose Utilization to Amino Acid Requirements of Staphylococcus aureus

Amino acid omitted from synthetic medium[a]	3A Susceptible: mg. of glucose consumed/100 mg. cells, Control	3A Susceptible: mg. of glucose consumed/100 mg. cells, ST 10 mg.-%	3A Susceptible: Ratio of glucose consumed Control/ST	3E Resistant: mg. of glucose consumed/100 mg. cells, Control	3E Resistant: mg. of glucose consumed/100 mg. cells, ST 100 mg.-%	3E Resistant: Ratio of glucose consumed Control/ST	Ratio of glucose consumption Strain 3A/Strain 3E
1. None	753	632	1.19	778	745	1.04	0.97
2. Alanine	729	0	∞	764	846	0.90	0.95
3. Glycine	1740	0	0	1551	1460	1.08	1.19
4. Proline	805	0	∞	547	0	∞	1.47
5. Aspartic	941	0	∞	0	0	0	∞
6. Glutamic	1290	0	∞	905	923	0.98	1.43
7. Methionine	1130	0	∞	718	0	∞	1.57
8. Phenylalanine	1170	710	1.65	624	859	0.73	1.88
9. Tyrosine	1070	0	∞	688	900	0.74	1.60
10. Histidine	802	0	∞	760	0	∞	1.05
11. Lysine	1150	664	1.73	825	883	0.93	1.39
12. Tryptophan	1270	0	∞	899	0	∞	1.41

[a] No growth was observed when valine, leucine, or arginine were omitted.

This latter inhibition of growth of the resistant strain 1E by 100 mg.-% sulfathiazole not only is completely abolished in the presence of glucose, but in fact there is a 16% acceleration of growth.

Strains 3A and 3E. The growth of the susceptible strain 3A (Table IV) was not inhibited by 0.5 mg.-% sulfathiazole in the absence of glucose. With the resistant strain 3E, the *ratio* of control growth to growth

of strain 3E in the presence of sulfathiazole is reduced from 2.84 in the absence of glucose to 1.19 in the presence of glucose. Since, in the absence of glucose, growth is inhibited 67% by 10 mg.-% sulfathiazole, and there is only 16% inhibition in the presence of glucose and 100 mg.-% sulfathiazole (Table IV), it would seem that the role of glucose is to supply energy and intermediaries for the reactions involved in the utilization of preformed amino acids and thereby bypass the inhibitory action of the drug. This role of glucose in the metabolism of strain 3E is not greatly inhibited by 100 mg.-% sulfathiazole. In contrast, 10 mg.-% sulfathizaole almost completely blocks this role of glucose for the susceptible strain 3A.

The Effect of Sulfathiazole on the Synthesis of Amino Acids

Strain 1A. In the absence of glucose, 0.5 mg.-% sulfathiazole completely blocks the growth associated with the synthesis of *glutamic acid*, and causes 50% inhibition of the growth dependent on the synthesis of *methionine*[4] and *lysine*. Ten mg.-% sulfathiazole, in the absence and presence of glucose, inhibits all growth 90–100%.

Strain 1E. In the absence of glucose, the growth dependent on the syntheiss of *alanine, proline, phenylalanine, histidine, lysine* is partially, and that dependent on *tryptophan* is completely, inhibited by 10 mg.-% sulfathiazole. In addition to these, 100 mg.-% sulfathiazole also inhibits the growth dependent upon the synthesis of *alanine, aspartic acid* and *glutamic acid*. In the presence of glucose, 100 mg.-% sulfathiazole inhibits 50% or more only the growth dependent on the synthesis of *glutamic acid, histidine, and tryptophan.*

These data show that the resistant strain (1E) in the presence of the drug and absence of glucose fails to synthesize *proline, phenylalanine, histidine,* and *tryptophan,* and in the presence of glucose *histidine* and *tryptophan,* all of which have in common a ring as a part of their structure. All the other amino acids can be synthesized by this strain under these conditions.

Strain 3A. In the absence of glucose, 0.5 mg.-% sulfathiazole inhibits 100% that growth which is dependent on the synthesis of *glutamic acid.*

[4] Studying the sensitivity to sulfanilamide of the synthesis of methionine, xanthine, serine, and pteroylglutamic acid, Winkler and de Haan (12) reported that the synthesis of methionine is the most sensitive to sulfanilamide and the synthesis of pteroylglutamic acid is the least sensitive of the series. In our study, the synthesis of glutamic acid is more sensitive than that of methionine, *etc.*

In the presence of glucose, 10 mg.-% sulfathiazole inhibits 50–100% the growth in all systems.

Strain 3E. In the absence of glucose, 10 mg.-% sulfathiazole inhibits 100% practically all of the *observed* growths which are dependent upon the synthesis of various amino acids. In the presence of glucose, 100 mg.-% sulfathiazole causes 50% or more inhibition of the growth dependent upon the synthesis of *proline, glutamic acid, methionine, phenylalanine, histidine,* and *tryptophan.*

This strain is so markedly deficient in synthetic ability in the absence of glucose that what little synthesis does occur is readily susceptible to sulfathiazole. This strain is capable of utilizing glucose for the synthesis of most of the amino acids. However, even in the presence of glucose, sulfathiazole inhibits the synthesis of those amino acids which contain a ring in their structure, and also the syntheses of glutamic acid and methionine.

Discussion of Results

The purpose of this study was to determine which amino acids are essential for growth of staphylococci, either susceptible or resistant to sulfonamides, and to note any differences with regard to amino acid requirements which may exist between the susceptible and resistant organisms in the presence and absence of a sulfonamide. Under the conditions of the experiments, the amount of growth obtained in the absence of any one of the amino acids may be considered as a measure of the synthetic ability of the organism in question with respect to the missing amino acid. The effect of glucose on the synthetic efficiency of the organism with respect to the individual amino acids in question has also been considered.

Comparison of the amounts of growth of the susceptible and resistant strains in the presence and absence of glucose or pyruvate will show that, in general, the resistant cells show a longer lag phase than do the susceptible cells. It has been observed and reported before that, associated with the development of resistance to sulfonamides, there is a decrease in the growth rate and metabolic activity of the resistant strain (3,8). The prolongation of the lag phase by the resistant cells is an indication that the initial critical factors which are required to initiate growth are synthesized at very much slower rates. This long lag phase can be reduced more that 60% in the presence of added vitamins [see Fig. 2, Ref. (9)]. This deficiency is related to lessened and modified biochemical activity on the part of the resistant cells.

It is evident from the data presented in the various tables that resistance to sulfonamides involves qualitative and quantitative metabolic differences between the paired drug-resistant and drug-susceptible strains.

Amino acid utilization by strain 3E (see last column, Table IV, growth *ratios*) is relatively sensitive to the drug, but when integrated with the glucose metabolism this deficiency is compensated for. In the case of the resistant strain 1E, a modified drug-resistant amino acid

TABLE V

Amino Acid Requirements of Staphylococcal Strains Susceptible and Resistant to Sulfonamides

See Tables III and IV for details

Basal amino acid medium		Basal amino acid medium +glucose	
Susceptible (1A)	Resistant (1E)	Susceptible (1A)	Resistant (1E)
Valine	Valine	Valine	Valine
Leucine	Leucine	Leucine[a]	Leucine[a]
Arginine	Arginine	Arginine	Arginine
	Glycine	Proline	Proline
	Glutamic acid[b]		Glycine[b]
	Methionine[b]		
Susceptible (3A)	Resistant (3E)	Susceptible (3A)	Resistant (3E)
Alanine	Alanine	Valine	Valine
Valine	Valine	Leucine	Leucine
Leucine	Leucine		Aspartic acid
	Aspartic acid		Glycine (partial)
	Glutamic acid		Proline[b]
	Methionine		Arginine[b]
	Histidine		
	Lysine		
	Phenylalanine[b]		
	Tyrosine[b]		
	Arginine[b]		
	Tryptophan[b]		

[a] Leucine is not a required amino acid if isoleucine is omitted from the medium and glucose is present.

[b] When these amino acids are limiting factors, only a trace of suboptimal growth occurs.

TABLE VI

Comparative Susceptibility and Resistance to Sulfathiazole of the Utilization and Synthesis of Amino Acids by Susceptible and Resistant Strains of Staphylococcus aureus.

Amino acids synthesized	Per cent inhibition of the utilization and synthesis of amino acids							
	S 1A		R 1E			S 3A	R 3E	
	No glucose	0.5% glucose	No glucose		0.5% glucose	0.5% glucose	No glucose	0.5% glucose
	100 γ ST/ml.	100 γ ST/ml.	100 γ ST/ml.	1000 γ ST/ml.	1000 γ ST/ml.	100 γ ST/ml.	100 γ ST/ml.	1000 γ ST/ml.
1. Complete medium	Complete inhibition	90–100 inhibition	−20	17	−15	89	64	19
2. Alanine			39	46	−35	100	Completely deficient in ability to synthesize any of the amino acids in the absence of ST	−21
3. Valine								
4. Leucine								
5. Leucine isoleucine					20			
6. Glycine					0	44		−20
7. Proline			69	77	0[b]	0[b]		0[b]
8. Aspartic acid			4	52	−7	100		0[c]
9. Glutamic acid[a]			0[a]	0[a]	50	100		90
10. Methionine			0[a]	0[a]	30	100		100
11. Phenylalanine			61	100	−27	90		55
12. Tyrosine			6	40	−25	100		24
13. Arginine			0[a]	0[a]	0[a]	0[a]		0[a]
14. Histidine			50	53	100	100		100
15. Lysine			44	45	−30	52		20
16. Tryptophan			100	100	100	100		100

[a] 5 γ of sulfathiazole/ml. medium inhibited completely growth of the susceptible strains in the absence of glutamic acid. Growth of the resistant strains in the absence of glutamic acid, methionine and arginine was either absent or suboptimal for an accurate measurement.

[b] In the presence of glucose and omission of proline, both the susceptible and resistant strains failed to grow.

[c] Resistant strain fails to synthesize aspartic acid, even in the presence of glucose.

metabolism is a critical factor in the phenomenon of resistance to the drug. Here also, glucose utilization is resistant to sulfathiazole.

It is evident from the data in Table III that the resistant strain 1E has suffered some impairment of ability to synthesize certain amino acids from other amino acids and make use of glucose to compensate partially or completely for these losses. The resistant strain 3E (Tables

IV, V and VI) shows a complete loss of ability to synthesize any of the amino acids in the absence of glucose. This strain was incapable of synthesizing *aspartic acid*[5] even in the presence of glucose. Since the syntheses of most amino acids are dependent on glucose metabolism, and since a certain number of these syntheses are susceptible to sulfathiazole, the enzymes involved in the metabolism of glucose and the tricarboxylic acid cycle supplying α-keto acids for amino acid synthesis must represent the most critical sites of sulfonamide action. This action appears to be related to the fact that the syntheses of several amino acids with the aid of glucose metabolism are insensitive to the drug.

In conclusion, these findings emphasize once again the point of view that sulfonamides interfere primarily with the functions of the oxidative enzymes of bacteria (10). For, in an amino acid medium deficient in purines and pyrimidines and vitamins such as riboflavin, pyridoxine, biotin, folic acid, *etc.*, their syntheses are dependent on the metabolism of amino acids and the utilization of the resulting metabolic products. Blocking of the metabolism of glucose and amino acids by sulfonamides would, therefore, constitute the primary site of drug action.

The loss of ability by the resistant cells to utilize one amino acid for the synthesis of another amino acid suggests that the enzymes involved in oxidative deamination, flavoproteins with or without coenzyme-linked dehydrogenase, may be impaired. As a consequence, the cells are incapable of deriving energy from amino acids and of producing deaminated compounds as a carbon source for synthetic purposes. As an alternate metabolic process, the organisms utilize glucose to obtain α-keto acids and energy for the synthesis of amino acids which are deficient in the medium. Here we have a clear demonstration of the role and nature of a drug-resistant alternate metabolic pathway in the mechanism of resistance to sulfonamides.

The concept has been advanced that cell enzyme proteins undergo configurational changes (10) with a lessened affinity for the drug. It has been experimentally demonstrated (11) that pneumococci, on becoming resistant to certain drugs, experience impairment of dehydrogenase activities. This impairment resides in the pneumococcal flavoprotein experiencing a greater degree of dissociation. It means that the coen-

[5] Strain 3E, which requires added aspartic acid for growth, was incapable of synthesizing aspartic acid in basal medium *via* added pyruvate, oxalacetate, α-ketoglutamate and fumarate with or without combinations with biotin, pyridoxal phosphate and $NaHCO_3$.

zyme group and the protein moiety of the enzymes of the resistant cell exercise decreased affinities for each other, which may also explain the loss of affinity by the protein for the drug. The degradative changes associated with the development of resistance which we have discussed can be interpreted in terms of the concept of configurational changes of the enzyme proteins.

Summary and Conclusions

A comparative study of the amino acid metabolism of two pairs of related strains of *Staphylococcus aureus* susceptible and resistant to sulfathiazole has resulted in the following findings and conclusions:

(1) Associated with the development of resistance there is (a) a prolonged lag phase during growth, and (b) partial or complete loss of ability to synthesize amino acids. These defects, in general, are compensated for by the metabolism of glucose. The resistant strain 3E is incapable of synthesizing aspartic acid, even in the presence of glucose.

(2) The utilization of preformed amino acids by one resistant strain, in contrast to that by the corresponding susceptible strain, is relatively insensitive to sulfathiazole. The resistance of the other strain is dependent on the simultaneous utilization of glucose.

(3) Though 5 γ of sulfathiazole/ml. does not inhibit the utilization of amino acids by the susceptible strains, it does, however, inhibit the synthesis of *glutamic acid* 100%, and that of *methionine* and *lysine* 50%. The syntheses by the resistant cells of *alanine, glycine, aspartic acid* (strain 1E), *phenylalanine, tyrosine* and *lysine*, in the presence of glucose, are not blocked by 1000 γ/ml. of sulfathiazole. The syntheses of other amino acids in the presence and absence of glucose are blocked to varying degrees by 100–1000 γ of sulfathiazole/ml.

(4) The relation of the above findings to the interference with the oxidative enzymes involved in the metabolism of glucose and the tricarboxylic acid cycle by sulfonamides is discussed.

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The Mechanism of Resistance to Sulfonamides. V. A Quantitative Study of the Alternate Metabolic Pathways Involving Tryptophan and Glucose Metabolism, and Aerobic and Anaerobic Growth Conditions in Relation to the Mechanism of Resistance [1]

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Introduction

It has been shown that the development of resistance to sulfonamides may be associated with degradative mutations resulting in deficient ability to utilize and synthesize amino acids in the absence of glucose and tryptophan (1). The fact that the presence of certain amino acids, such as tryptophan and histidine (and to a lesser extent, others) and of glucose abolish this deficiency in the resistant strain (2,1), would indicate that the combined metabolism of glucose and these amino acids by the resistant cells differs from that by the susceptible cells and thus is a critical factor in the mechanism of resistance to sulfonamides. These facts suggested the present study of the quantitative relation of the utilization of amino acids and glucose to the resistance mechanism; tryptophan was chosen as a representative amino acid.

Experimental Methods

Organisms

As indicated in the tables the same organisms were used in this study as in the preceding paper (1).

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Culture Media

The basal medium was a $M/30$ phosphate solution (pH 7.35) of 1% casein hydrolyzate (SMACO, vitamin free), 8.0×10^{-5} M of cysteine hydrochloride, 2.5×10^{-4} M of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 3.3×10^{-4} M of $MgSO_4 \cdot 7H_2O$ and 1 γ/ml. each of thiamine chloride and nicotinamide.

In addition to the basal medium, 3 modifications were used: (1) basal medium plus 20 γ of *l*-tryptophan/ml.; (2) basal medium plus 0.5% glucose; and (3) basal medium plus 0.5% glucose and 20 γ of *l*-tryptophan/ml.

Concentration of Sulfathiazole

The concentration of the drug used was 50 mg.-% (500 γ/ml.) in each of the above 4 media.

Inoculum

The inoculum was prepared as in the preceding paper (1) except that the final inoculum was 5×10^6 organisms/ml. of medium.

Growth

This was determined as in the preceding paper (1).

Quantitative Determination of Tryptophan

A macro (3) and micro (4) colorimetric method were both used for tryptophan determinations of: (a) the amount of tryptophan in the cells harvested at various periods; and (b) the amount of added tryptophan consumed from the media during growth of the cells.

a. Determination of Tryptophan Content of the Cells

Macro Method. The amount of tryptophan in the cells harvested at various periods was determined in the following manner: the cells harvested from the medium were washed 3 times and finally suspended in water. An aliquot of this aqueous suspension (40 mg.) of the cells was dried in a 15 ml. weighing bottle to constant weight at 106°C. The dried cells were then digested in 5 ml. of 5 N NaOH in a 56°C. water bath for 1 hr. The samples following digestions were analyzed for tryptophan according to the macro method.

Micro Method. The washed samples of the cells for this method were prepared as in the above-described method except that, following the washing with water, the cells were washed successively with absolute alcohol and ether and then dried at 106°C. to constant weight. Five mg. of the dried sample were digested in 1 ml. of 5 N NaOH in a 56°C. water bath for 1 hr. and then analyzed for tryptophan according to the micro method (4).

b. *Determination of the Consumption of Added Tryptophan by the Growing Cells*

This determination was made by duplicate analysis at various times of cell-free samples of the culture fluid by the macro and micro methods for tryptophan. Tryptophan consumed by the organism would then be represented by the difference in tryptophan content of the medium at 0 hr. and at the time of subsequent analysis. The proper controls were used in all determinations.

Glucose

Glucose was determined on cell-free samples of the culture fluid by the Folin-Wu method (5).

Presentation and Discussion of Results [2]

The Antagonistic Effect of Preformed Tryptophan on the Inhibitory Actions of Sulfathiazole

The results in Table I show the behavior of a pair of related strains of *Staphylococcus aureus* susceptible and resistant to sulfathiazole.

Susceptible Strain. In the absence of preformed tyrptophan and glucose, the growth of this strain related to the synthesis of tryptophan is completely inhibited by sulfathiazole.[3] The presence of either preformed tryptophan or glucose only partially relieves this inhibition. When both glucose and preformed tryptophan are present, a further reduction in the inhibition by sulfathiazole occurs.

Resistant Strain. The growth related to the synthesis of tryptophan by this strain is also inhibited by sulfathiazole. When, however, preformed tryptophan is present in the medium, the inhibition of growth by sulfathiazole is completely abolished, which indicates that the preformed tryptophan antagonizes sulfathiazole (2,6).

The two strains differ, therefore, in the following respects: (a) In the presence of glucose and sulfathiazole, but in the absence of preformed

[2] The results of the single experiments presented in this paper are in each case representative of a group of experiments, the results of which were qualitatively similar.

[3] Five hundred ml. of the sterile-filtered culture fluids of the susceptible and resistant cells which were grown for 48 hr. in basal media containing glucose, but no preformed tryptophan, were analyzed for tryptophan. They were concentrated *in vacuo* at 5 mm. pressure and 20°C. in an atmosphere of nitrogen. The concentrates were, with several washings, 35 ml. each. Using the micro method, no tryptophan was detected. These findings indicate that both the susceptible and resistant cells make complete use of the tryptophan synthesized.

tryptophan, there is a 37% growth of the susceptible cells and none whatsoever with the resistant cells. Under these conditions, the synthesis of tryptophan by the susceptible cells is less sensitive to sulfathiazole than is this synthesis by the resistant cells; (b) On the other hand, preformed tryptophan only partially counteracts the inhibition of the growth of the susceptible cells by sulfathiazole and completely counteracts that of the resistant cells. [See further, (1).]

TABLE I

Tryptophan Metabolism by Staphylococcus aureus Strains 1A and 1E[a b]

Composition of growth medium	Susceptible 1A				Resistant 1E			
	Cells/ 100 ml.	Tryptophan/100 mg. cells			Cells/ 100 ml.	Tryptophan/100 mg. cells		
		Consumed	Found	Per cent consumed, not accounted for		Consumed	Found	Per cent consumed, not accounted for
	mg	γ	γ		*mg*	γ	γ	
BM	7.4	0	393		4.0	0	430	
BM+Tr	7.9	760	491	55	4.6	652	457	42
BM+Tr+ST	2.3	0	335		5.5	1091	304	259
BM+Gl	36.8	0	368		40.4	0	396	
BM+Gl+ST	13.8	0	394		0	0	0	
BM+Gl+Tr	49.7	845	453	86	41.7	600	488	43
BM+Gl+Tr+ST	23.0	1044	419	150	44.2	943	492	91

[a] Growth period 72 hr.

[b] Inoculum size = 5×10^6 organisms/ml.

BM = basal medium; ST = sulfathiazole; Tr = tryptophan; Gl = Glucose.

Tryptophan does not augment growth to a significant degree. Growth of the resistant strain in the presence of glucose, with or without tryptophan, is 10-fold greater than in the tryptophan-free basal medium. Sulfathiazole completely blocks in the absence, but completely fails to block the growth in the presence, of preformed tryptophan.

Under the above conditions, there is from 91% to 259% more tryptophan consumed in the presence of sulfathiazole than can be accounted for by the tryptophan content of the cells. This extra consumption of tryptophan does not increase growth. In this connection, a previous suggestion (2) might be recalled that tryptophan metabolism yields *o*-aminobenzoic acid and kynurenine [see footnote 1 in the preceding

article (1)]. It is possible that sulfathiazole accelerates the formation of these products from tryptophan. Furthermore, it is not at all unlikely that a breakdown product of tryptophan supplies an intermediary for a drug-insensitive pathway.

In conclusion, the data presented in Table I show that, in the basal amino acid medium containing tryptophan, the failure of sulfathiazole to block the growth of the resistant cells is associated with a consumption of a considerably increased amount of preformed tryptophan. This effect is exclusively peculiar to the resistant cells. These data show an

TABLE II

A Study of the Kinetics of the Utilization of Tryptophan and Glucose by Staphylococcus aureus[a]

Growth	Susceptible strain (1A)								Resistant strain (1E)							
	mg. of cells/100 ml. culture		mg. glucose consumed/100 mg. cells		γ Tryptophan/100 mg. cells				mg. of cells /100 ml. culture		mg. glucose consumed/ 100 mg. cells		γ Tryptophan/100 mg. cells			
					Consumed		Found						Consumed		Found	
	Control	ST	Control	ST	Control	ST	Control	ST	Control	ST	Control	ST	Control	ST	Control	ST
hr.																
16	20.7	3.7	725	1222	628	0	400	400	10.1	13.3	742	145	495	975	400	440
24	38.2	6.0	786	1254	654	0	420	430	26.2	27.0	1106	741	532	940	470	490
40	43.7	13.8	938	1304	778	579	420	420	34.1	34.1	1366	1028	585	970	480	480
65	48.8	34.1	872	1187	697	646	430	440	35.0	35.0	1413	1110	600	943	480	500

[a] The medium used is that described in the experimental section. 0.5% glucose and 20 γ of tryptophan/ml. have been added. The drug (ST) concentration is 50 mg.-%.

interrelated glucose and tryptophan metabolism with respect to drug-susceptibility and drug-resistance. Studies of the utilization of glucose and tryptophan during growth should be helpful in clarifying the respective functions of these two substances in the resistance phenomena.

Studies of the Utilization of Glucose and Tryptophan during Growth

Table II contains the measurements of the utilization of added tryptophan and glucose. According to these results, whenever utilization of added tryptophan is observed, the rate of growth of the susceptible

cells increases with simultaneous decline in the degree of inhibition of growth. Neither tryptophan nor glucose alone are of sufficient aid in overcoming the inhibition resulting from the action of sulfathiazole, but in combination they are effective. The inhibition of growth of the susceptible strain has dropped to 30% at 65 hr. (Table III). From Table II it can be seen that at this time the susceptible organism is utilizing added tryptophan in normal amounts with a slight decrease in the amount of glucose consumed per unit weight of cells. These facts

TABLE III

Alternate Metabolic Pathways of S. aureus

Data with respect to	Growth period	Per cent change with ST[a]	
		Susceptible (1A)	Resistant (1E)
	hrs.		
Growth	16	82	−36
	24	84	−6
	40	68	0
	65	30	0
Tryptophan utilization	16	100	−97
	24	100	−77
	40	26	−66
	65	7	−57
Glucose utilization	16	−68	80
	24	−60	49
	40	−39	33
	65	−36	21

ST = 50 mg.-%.

[a] Figures indicate percentage decrease unless preceded by a minus sign, in which case they are percentage increase.

would indicate that the susceptible strain has begun to make use of an alternate amino acid metabolism, which is less sensitive to the action of sulfathiazole. The use of the latter metabolism permits a more efficient utilization of glucose for growth, indicated by a smaller amount of glucose consumed per unit weight of cells.

In contrast, survey of the growth figures involving the resistant strains shows clearly that the utilization of added tryptophan is not

blocked. Associated with the development of resistance there is an emergence of a resistant type of tryptophan (utilization) metabolism to which the glucose metabolism plays a secondary role.

A type of alternate metabolic pathway involving glucose and amino acid metabolism in relation to resistance was suggested by Sevag and Green (2) and is discussed at length by Sevag (7). The measurements in Table II indicate that both the susceptible and resistant cells resort to the use of alternate metabolic pathways to overcome, partially or wholly, the inhibitory effects of the drug. This is indicated by the analysis of the data presented in Table III. On the basis of these data it would seem that the blocking of the utilization of tryptophan by the drug-susceptible cells may be compensated for by an accelerated metabolism of glucose, thus permitting a limited amount of growth of the susceptible strain. The above apparent acceleration of the metabolism of glucose in the presence of sulfathiazole by the drug-susceptible strain is not associated with a proportional increase in the degree of growth and may indicate that sulfathiazole is accelerating the breakdown of glucose, but not permitting the utilization of the products for synthetic processes.[4]

These relationships are reversed for the drug-resistant organisms. Here, not the tryptophan utilization but the glucose utilization is blocked. Thus, the resistant cells bypass the blocking of the glucose utilization by the drug. This bypassing occurs through the use of the drug-accelerated utilization of tryptophan, which permits uninhibited, even accelerated, growth in the presence of the drug.

The Relation of Glucose Consumption to the Synthesis of Amino Acids by the Resistant Strain as Further Evidence of an Alternate Metabolic Pathway

Steers and Sevag (1), in a study of the amino acid metabolism of *Staphylococci* susceptible and resistant to sulfathiazole, presented evidence to show that the metabolism associated with the development

[4] This type of acceleration of the breakdown of glucose and other substrates by an inhibitor has been observed previously. For discussions of the subject see Clifton (8), Sevag (7), McElroy (9). Similarly, the acceleration in the presence of sulfathiazole of the consumption of tryptophan by the resistant cells in excess of the amount of tryptophan found in the cells may or may not serve for other synthetic processes. A possible deviation from the synthesis of histidine in the presence of sulfonamides may take place, and give a structurally related product which was characterized by Shive *et al.* (10) as 5(4)-amino-4(5)-imidazolecarboxamide.

of resistance was concerned with the utilization of amino acids, with or without associated glucose metabolism, by the resistant strains. The evidence presented here serves to emphasize this point.

The data in Fig. 1 show that, in the absence of the drug, the resistant organism consumes a greater amount of glucose per mg. of cells than does the susceptible organism in the synthesis of an amino acid. Ten mg.-% of sulfathiazole inhibits completely the growth of the susceptible strain dependent upon both the utilization and synthesis of amino acids. The growth of the resistant strain dependent upon the synthesis

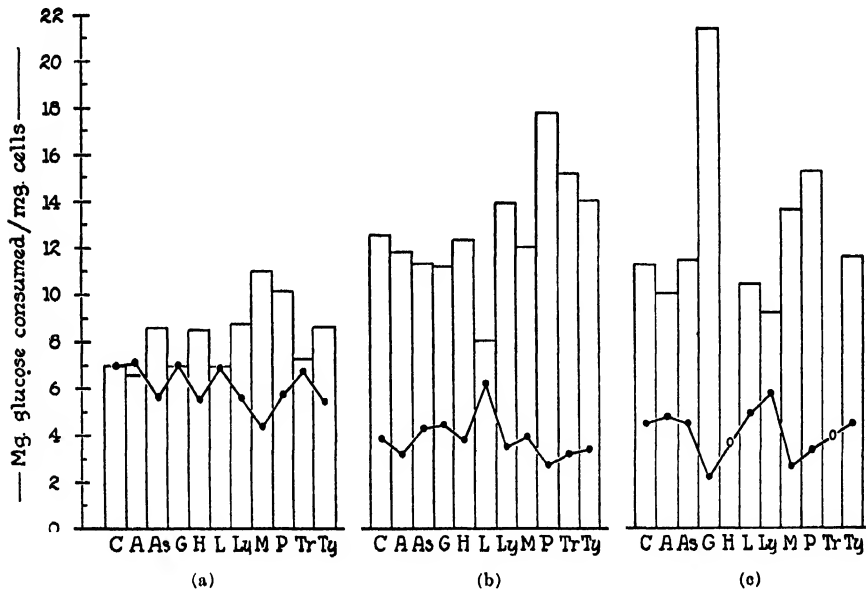

FIG. 1. Consumption of glucose associated with the growth dependent upon the synthesis of amino acids.

(a) = Susceptible strain (1A) in the absence of ST. The synthesis of amino acids by strain (1A) was completely inhibited by 10 mg.% of ST.

(b) = Resistant strain (1E) in the absence of ST.

(c) = Resistant strain (1E) in the presence of 100 mg.% ST.

Blocks represent milligrams of glucose consumed per milligram of cells during growth dependent on the synthesis of the respective amino acids. The solid points in the curve correspond to the milligrams of cells grown per 10 ml. medium. Open circles represent complete inhibition of growth by ST.

C. Complete medium	G. Glutamic acid	Ly. Lysine	Tr. Tryptophan
A. Alanine	H. Histidine	M. Methionine	Ty. Tyrosine
As. Aspartic Acid	L. Leucine	P. Phenylalanine	

of *tryptophan, histidine, glutamic acid, methionine,* and *leucine,* is inhibited to varying degrees by sulfathiazole. On the other hand, the growth of this strain which is dependent upon the syntheses of *lysine, alanine, tyrosine, aspartic acid and phenylalanine* is increased in the presence of sulfathiazole. *Inhibition by the drug of growth dependent on the synthesis of amino acids is associated with increased glucose consumption per mg. of cells,*[5] *whereas acceleration by the drug of growth dependent upon synthesis of amino acids results in a decreased glucose consumption per mg. of cells.* Where growth of the resistant strain does occur in the absence of an amino acid and presence of the drug *the glucose consumption per mg. of cells is greater than that by the susceptible strain in the absence of the drug* (Fig. 1). Both the susceptible and resistant cells during growth consumed the same amount of glucose per unit volume; however, the glucose consumed by the resistant cells results in less growth per unit volume (Fig. 1). This would indicate that, in the development of resistance, there has been degradation either in the character or content of those enzymes which are concerned with the metabolism of glucose related to amino acid metabolism. This is in part evident from Table IV, which shows that the resistant organism is a strict aerobe and its glucose metabolism is, therefore, aerobic and different from that of the susceptible cells.

If we consider the data in Fig. 1, we will see that, whenever the synthesis of an amino acid is inhibited, although there is a greater amount of glucose consumed per unit weight of cells, the growth is sparse. In contrast, whenever the synthesis of an amino acid is free from inhibition, there is less glucose consumed per unit weight of cells, with better growth. In other words, in the former case the economy of the cells with respect to glucose is wasteful; in the latter case the economy is more efficient with respect to growth.

The Relation of Glucose-Glutamic Acid Metabolism to the Mode of Drug Action and the Resistance Mechanism

The growth of the resistant strain dependent on the synthesis of glutamic acid by way of glucose metabolism is inhibited 50% by 1000

[5] The data presented in Fig. 1 show that, in the presence of sulfathiazole, the growth of the resistant cells dependent on the synthesis of glutamic acid involved the utilization of a two-fold greater amount of glucose when compared with that consumed in the drug-free system. This represents the largest amount of glucose consumed in the synthesis of any of the amino acids studied.

γ sulfathiazole/ml. During this growth, twice as much glucose is consumed as in the absence of sulfathiazole. For this strain, this is the greatest amount of glucose consumed per unit weight of cells. The susceptibility of the synthesis of glutamic acid by the susceptible cells is carried over in part to the resistant cells. Sulfathiazole is interfering with the enzymes involved in the formation of intermediaries concerned in the synthesis of glutamic acid. As a consequence of this action of the drug, the glucose metabolism is being diverted into pathways which do not yield optimal growth, which might account for the two-fold greater consumption of glucose in the presence of the drug. In view of the fact that, in general, proteins contain a higher percentage of glutamic acid than of the other amino acids, and because of the critical role of glutamic acid in transamination and, therefore, in the syntheses of new amino acids, the above considerations with respect to the glucose-glutamic acid metabolism become increasingly significant in the mode of drug action and of the resistance mechanism.

The Effect of Aerobiosis and Anaerobiosis upon the Growth of Staphylococcus aureus Susceptible and Resistant to Sulfathiazole

The evidence presented thus far indicates that the susceptible and resistant organisms make use of alternate metabolic pathways to overcome the action of the drug. A comparison of the amount of glucose consumed by the susceptible strain and the resistant strain in the absence of sulfathiazole shows that the latter strain consumes much more glucose per unit weight of cell than does the former. However, in the presence of the drug the susceptible cell consumes an increased amount of glucose, which is approximately equal to that consumed by the resistant cell in the absence of the drug. The susceptibility of glucose metabolism to drug action in one strain and resistance in the other indicates a difference in the nature of this metabolism in the two strains. Determination of the oxygen requirements for growth of the susceptible and resistant strains in the presence and absence of the drug provides a partial answer as to how they differ.

From the data in Table IV it is evident that the susceptible strain 1A utilizes both aerobic and anaerobic metabolisms for growth, whereas the resistant strain 1E utilizes only the aërobic metabolic mechanism, because it is either deficient in the minimal number of anaerobic

metabolic mechanisms required for growth or possesses no anaerobic metabolic mechanisms at all. This would be evidence to support the viewpoint of Sevag (7) that, associated with resistance, there are degradative mutations which result in the loss of one or more enzyme components.

Three observations concerning the suspeptible strain can be related to explain the action of sulfathiazole upon the susceptible strain and in what way this organism may utilize glucose partially to overcome this inhibition. (1) *Aerobically*, in the presence of 50 mg.-% sulfathiazole the growth is 60% of that occurring in the absence of the drug; (2) *anaerobically*, the growth of this strain in the absence of sulfathiazole is 40% of the growth occurring in an aerobic atmosphere in the absence of the

TABLE IV

Effect of Aerobiosis and Anaerobiosis upon the Growth of Staphylococcus aureus 1A (Sulfonamide-susceptible) and 1E (Sulfonamide-Resistant) Growth period 96 hr.[a]

Strain	Aerobic growth		Anaerobic growth	
	mg. cells/10 ml.		mg. cells/10 ml.	
	Control	ST	Control	ST
1A (susceptible)	5.7	3.5	2.4	0
1E (resistant)	4.2	4.4	0.2	0.5

[a] The medium used is that described in the experimental section. 0.5% glucose and 20 γ of tryptophan have been added. The drug (ST) concentration is 50 mg.-%.

drug; and (3) the growth of the susceptible strain which does occur in an anaerobic atmosphere is 100% inhibited by the drug. These considerations would indicate that: (a) since sulfathiazole completely eliminates the anaerobic growth of the susceptible organism, the effect of the drug upon the growth in the presence of air would be to eliminate the fraction of growth due to anaerobic metabolism; and (b) although, in the absence of the drug, the susceptible organism is capable of a combined and coordinated aerobic and anaerobic metabolism of glucose for growth, the drug blocks the anaerobic metabolism of glucose necessitating dependence of the organism upon the aerobic metabolism of glucose. The effect of the sulfonamides then seems to be the suppression

of one or more anearobic mechanisms of the susceptible organism, resulting in an inhibition of growth. This would appear to be in agreement with the observation of Sevag, Richardson and Henry (11) that sulfonamides do not inhibit the oxygen consumption by *Staphylococcus aureus.*

This may appear to represent an inconsistency in relation to previously reported data on the inhibition of oxidative mechanisms by sulfonamides. However, a consideration of the role of oxidative enzymes in aerobic and anaerobic metabolism would indicate that the inconsistency is more apparent than real.

The fact that the resistant strain aerobically is not inhibited by sulfathiazole, whereas anaerobically it fails to grow in the absence of the drug, would indicate that the development of resistance by the organism in the presence of the drug results not only in the loss of one or more anaerobic mechanisms, but also in an alteration of the aerobic metabolic mechansims, since these aerobic mechanisms are more resistant to drug action than the corresponding mechanisms of the susceptible strain.

Summary and Conclusions

Four quantitative determinations have been made at various time intervals; (1) the weight of organisms per unit volume of culture medium, (2) the amount of tryptophan in the cells harvested from the media, (3) the amount of added tryptophan consumed per unit weight of cells, and (4) the amount of glucose consumed per unit weight of cells. The results obtained show that both the sulfonamide-susceptible and -resistant organisms make use of alternate metabolic pathways to circumvent the action of the drug. These alternate metabolic pathways involve the participation of glucose and tryptophan in a manner distinctive, respectively, for the resistant and susceptible cells. Tryptophan functions as an antagonist of sulfathiazole in the metabolism of the resistant cells.

The inhibition by the drug of growth dependent on the synthesis of certain amino acids is associated with increased glucose breakdown per mg. of cells. On the other hand, the acceleration by the drug of growth dependent upon synthesis of amino acids (phenylalanine, alanine, tyrosine, and lysine) results in a decreased glucose consumption per mg. of cells. In this connection, the role of glucose-glutamic acid metabolism in relation to the mode of action of sulfonamides and the resistance mechanism is discussed.

A study of the oxygen requirements of the drug-susceptible and drug-resistant strains leads to two conclusions: (1) Sulfathiazole acts upon the anaerobic metabolism of the susceptible organism; and (2) the development of resistance has resulted in degradative mutations as manifested by the fact that the resistant organism has become a strict aerobe.

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The "Browning" Reaction of Proteins with Glucose [1]

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Introduction

The development of a brown color in solutions containing mixtures of amino acids and reducing sugars was first described by Maillard (1). That proteins may react with reducing sugars in similar fashion was indicated clearly by Ramsey, Tracy and Ruehe (2) with casein and glucose. Subsequently, a number of other investigators (3–11) studied systems containing both proteins and reducing sugar. In most of these studies, dry mixes were used since non-enzymatic deterioration of dried food products during storage was a problem of practical importance that required solution. Space does not permit review of the considerable amount of important and useful information that was acquired by these techniques (for references, see 2–14).

In an extensive wartime program on egg dehydration at this laboratory, it was found that the "browning" reaction with proteins occurred also in solution, thus simplifying reaction rate measurements, isolation procedures, *etc.* It was the object of the present investigation to determine the effects of varying conditions on the rate of browning, the properties of the "browned" protein derivatives, and the protein groups involved in the reaction.

Experimental

Materials and Methods

Crystalline bovine serum albumin (BSA) from a commercial source was used for most of the experiments. Other proteins and their donors were crystalline lysozyme

[1] This work benefited from association with the cooperative project on non-enzymatic browning sponsored by the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces.

[2] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

(G. Alderton), protamine sulfate (Eli Lilly and Co.), edestin (D. M. Greenberg), guanidylated human serum albumin (W. R. L. Hughes, Jr.), and ovomucoid (H. Lineweaver). The tyrosine-formaldehyde preparation had been polymerized in acid solution and contained approximately 5% amino nitrogen.[3] A second polyamino polymer was obtained by the reaction of polymethyl polyglutamic ester with hexamethylene diamine (see Preparative). Polyglutamine has been previously characterized (15). Aminoacetyl bovine serum albumin was prepared by reaction with acetic anhydride in cold sodium acetate solution (16). The product used contained 0.08% amino nitrogen and acetyl groups corresponding to the difference in the number of amino groups in BSA and its derivative.

The procedure for measuring the development of color was typically as follows: 1 g. of protein was dissolved in a mixture of 2 ml. of 3.4 *M* potassium phosphate buffer (pH 7.6) and 10 ml. of 37.5% glucose solution. A control solution contained no protein. After the necessary time intervals, 1 ml. samples of each were diluted to 10 ml. Transmission data were obtained with a Coleman Model 11 spectrophotometer[4] at 500 mμ. The control solutions, used to adjust the instrument for 100% transmittance, developed very little color compared to those containing protein. No attempts were made to regulate the atmosphere in contact with the solutions, since experiments with glycine-glucose solutions had shown that the rate of browning was as rapid in the absence of air (high vacuum) as in the presence of pure oxygen (6).

Products were isolated by dialysis against continued changes of distilled water, then dried by lyophilizing. They were allowed to come to equilibrium with atmospheric conditions before being analyzed. For moisture content, samples were dried to constant weight *in vacuo* in an Abderhalden drier heated with boiling acetone (56°C). Total nitrogen was determined by a micro Kjeldahl procedure recently shown not to require the lengthy digestion times often recommended (17). Amino nitrogen was determined by the Van Slyke manometric method (18), with a 15-min. reaction period. An error of small magnitude, probably due to the reducing activity of browned protein-glucose products, was introduced when these products were analyzed.[5] The ninhydrin method of Harding and MacLean (19), although satisfactory for BSA, could not be used with the glucose derivatives, since it gave amino nitrogen values 2–3 times as high as did the Van Slyke method, possibly also as a result of the reducing activity.

Tryptophan was estimated by the method of Horn and Jones (20) without hydrolysis. Total guanidyl groups were determined in acid hydrolyzates (6 *N* HCl; 18 hr.;

[3] Olcott, H. S., manuscript in preparation.

[4] Mention of equipment by name does not constitute endorsement over similar equipment available from other manufacturers.

[5] One g. of proline was dissolved in a mixture containing 2 ml. of 3.4 *M* phosphate buffer (pH 8.0) and 10 ml. of 37.5% glucose solution. The resulting pH was 7.3. Half of this solution was heated for 30 min. at 100°C., whereupon it turned light brown. Heated and unheated solutions were analyzed by the Van Slyke procedure. The first evolved nitrogen equivalent to 2.8% of that present in the proline; the second, only 0.16%, a negligible amount. A control glucose solution heated in the absence of proline remained water-white and contained no apparent amino nitrogen. When the same treatment was applied to an equimolar solution of alanine, its apparent amino nitrogen content was reduced by 39%.

oil-bath temperature, 120–130°C.) by the method of Brand and Kassell (21); total acid and basic groups by a dye method (22); free glucose, by Somogyi's method (23); and bound glucose by an orcinol procedure (24). The reducing capacity of the protein derivatives against $K_3Fe(CN)_6$ was determined by the method of Mirsky and Anson (25).

The amount of formaldehyde liberated by reaction with periodic acid was determined by a modification of the technique used by Boyd and Logan (26) for the determination of serine. One hundred mg. of protein in a small distilling flask was dissolved in 15 ml. of water, to which were then added 3 drops of methyl red indicator, 4 ml. of 25% potassium arsenite, 2.5–2.8 ml. of 0.5 *M* periodic acid (the last 0.5 ml. dropwise until the solution was acid to methyl red), and 15 ml. of water. The solution was then distilled until only 5 ml. remained. Formaldehyde was determined in the distillate with chromotropic acid (27). This technique, when applied to gelatin, gave values of 0.174 and 0.179% formaldehyde, compared to the value of 0.179% found by Desnuelle and Antonin (28).[6]

All pH determinations were made with the glass electrode standardized at pH 4.0 with 0.05 *M* phthalate buffer.

Results

Bovine serum albumin (BSA) was used to study the effects of temperature, pH, and other variables on the rate of browning. Its ready solubility in water, resistance to denaturation, availability, and higher content of amino nitrogen (approximately 1.2%) than that of most other proteins were advantageous.

Effect of Temperature

The shape of the browning curve and the effect of temperature on the reaction are shown in Fig. 1. There is a short induction period followed by a protracted period during which the increase in color is linear with time. When the logarithms of the rates of reaction based upon the linear portion of the curve are plotted against the reciprocals of the absolute temperatures (Fig. 2), a straight line is obtained; evidence that the same reaction mechanism occurs in the temperature range of 25° to 65°C. At 70°C. the solutions set to gels preventing rate measurements. The apparent activation energy, calculated from these data, is 30.3 kg.-cal., a value somewhat higher than that observed with many organic reactions, but of the same magnitude as those recorded for the browning of apricots (29) and dried vegetables (5).

Effect of pH

The browning reaction is accelerated by increases in alkalinity as shown in Fig. 3, as was predictable from the work of previous investigators. The linearity of the plot of logarithm of reaction rate against pH suggests that hydroxyl ion is a catalyst (30). The one value that did not fall on the line was obtained in acetate buffer (3.4 *M*

[6] Desnuelle and Antonin (28) have used this value as a measure of the hydroxylysine content of gelatin.

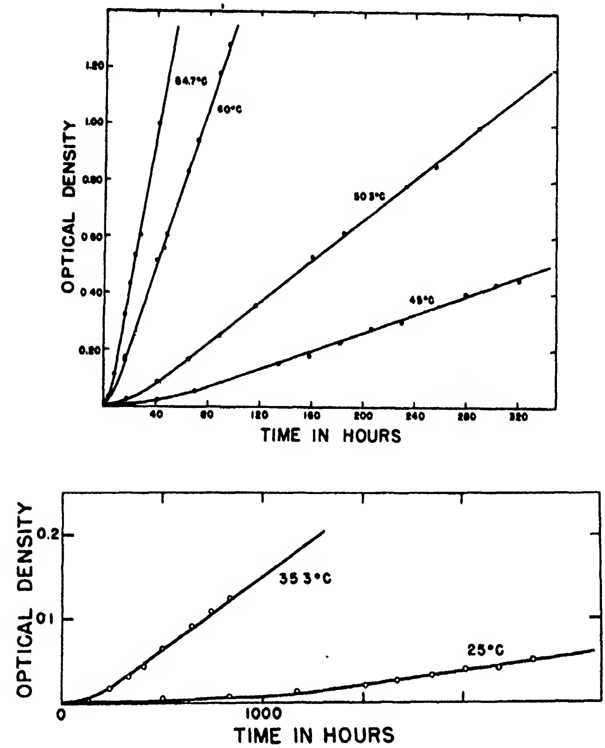


Fig. 1. Effect of temperature on rate of browning of bovine serum albumin-glucose solutions (bovine serum albumin 8%, glucose 30%, phosphate 0.55 M, pH 7).

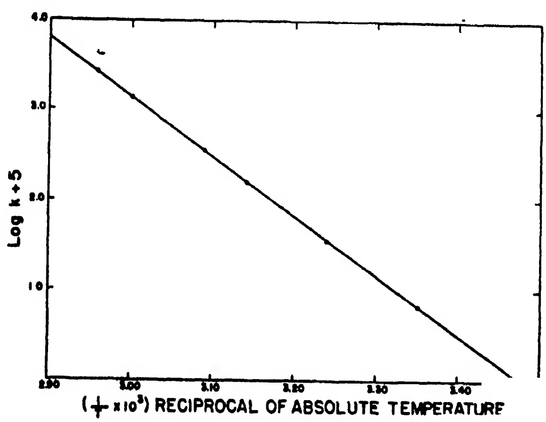


Fig. 2. Plot of logarithm of the rate of browning of bovine serum albumin-glucose solutions (pH 7.0) against the reciprocal of the absolute temperature (*cf.* Fig. 1).

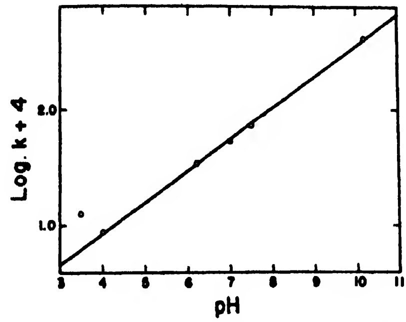


FIG. 3. Effect of pH on rate of browning of bovine serum albumin-glucose solutions at 53°C. (see text).

acetic acid). The other runs were without added buffer (pH 4.0) or in phosphate buffer (pH 6.2, 7.0, 7.5, 10.2). The solution made up to pH 4.0 by careful adjustment with 0.1 N HCl was self-buffered. After 29 days at 53°C., the measured pH was 3.95. Solutions of BSA (8%) and glucose (30%) in phosphate buffers (0.62 M) of pH 2.0, 3.0 and 4.0 gelled within a few hours, both at 53° and 45°C.[7]

Effect of Protein and Glucose Concentration and Added Substances

The effects of varying protein and glucose concentration are shown in Table I. Unless glucose is present in concentrations higher than 10% or higher, gelation interferes with color measurement. The rate of browning appears to be dependent upon, but not directly proportional to, protein concentration except within the range 6–9%. It was thought that the role of glucose in preventing gelation might be similar to the known effects of concentrated solutions of sucrosé and glycerol in preventing protein denaturation. In accord, substitution of these reagents for most of the glucose did prevent the formation of gels, and permitted measurement of the rate of browning in dilute glucose solutions. The inclusion of sucrose or glycerol appeared to inhibit somewhat the rate of browning.

Since sulfite is often used to inhibit browning in foods, it was considered of interest to determine whether it would also inhibit the BSA-glucose reaction. However, a solution containing 0.04 M $NaHSO_3$ (8% BSA, 30% glucose, 0.6 M phosphate) set to a gel within a few hours. There was no apparent inhibition of browning. Gelation also occurred when the solutions contained 15% sodium salicylate and 15 or 30% urea in addition to the sulfite and glucose.

Traces of copper accelerate the browning reaction of amino acids with glucose.[8] In one experiment it was found that copper in a concentration of 0.02% (10.0 mg.

[7] The following additional observations were made on BSA denaturation: Solutions of BSA (8%) at pH 2, 3, or 4 in the presence of 0.55 M potassium phosphate buffer set to firm gels within a few hours at 45°C. At pH 5 a similar solution remained clear at 45°C. for most of a period of 14 days, then developed a slight haze.

[8] Unpublished observations.

$CuSO_4 \cdot 5H_2O$ in 12.5 ml. total volume) accelerated the rate of browning of BSA in glucose solution but did *not* appear to affect appreciably the rate of disappearance of amino groups. The transmissivities at 2 days and 5 days were, for the sample containing copper, 43 and 14%, respectively; and for the control, 73 and 22%. The amino nitrogen values were 0.45% and 0.43% for the experimental and control samples at 2 days, and 0.31% and 0.32% at 5 days.

Phosphate buffer did not appear to have any specific effect on the rate of the reaction. The rate of browning was essentially the same when the buffer was phosphate, carbonate, or veronal at 0.1 *M* concentration (Table II). Solubility of the veronal buffer was the limiting factor with respect to the concentrations of buffer used in this experiment.

TABLE I

Effect of Concentration of Components on Rate of Browning of BSA-Glucose Solutions[a]

Run	BSA concentration	Glucose concentration	Sucrose concentration	Percentage transmission					
	per cent	*per cent*	*per cent*	*hr.*	*per cent*	*hr.*	*per cent*	*hr.*	*per cent*
A	2[b]	30	0	52	83	120	54	168	46
	4[b]	30	0	52	74	120	39	168	28
	6[b]	30	0	52	66	120	28	168	17
	8[b]	30	0	52	58	120	20	168	10
	9[b]	30	0	52	56	120	18	168	9
B	8	2[c]	30	80	77	211	48	475	26
	8	5[c]	30	80	70	211	33	375	14
	8	10[c]	30	80	65	211	25	475	9
C	8	10	0	73	43	215	8	—	—
	8	30	0	74	58	210	14	—	—
	8	50	0	74	55	210	12	—	—

[a] In 0.55 *M* phosphate buffer, initial pH, 7.3. Sucrose was required at the lower glucose concentrations to prevent gelation during the course of the run. Parallel runs, in which 30% glycerol rather than 30% sucrose was used, gave color intensities between those of the control and those containing sucrose. Temperature, 53°C., air oven. The data shown in the separate runs are not directly comparable with each other because of fluctuations in oven temperature. The data shown in Figs. 1 and 2 were obtained with experiments run in constant temperature water baths.

[b] The products isolated after 168 hr. of reaction had nitrogen contents of 13.0, 13.2, 13.2, 13.1, 13.1%, and amino nitrogen contents of 0.17, 0.15, 0.20, 0.12, 0.15%, dry basis.

[c] Amino nitrogen values obtained from samples isolated after 619 hr. were 0.48%, 0.26%, and 0.18% for the 2%, 5%, and 10% glucose solutions, respectively.

TABLE II

Effect of Buffers on Development of Browning in BSA-Glucose Solutions at 53°C.[a]

Buffer	Original pH	Final pH	Percentage transmission[b]			
			113 hr.	209 hr.	377 hr.	689 hr.[c]
			per cent	*per cent*	*per cent*	*per cent*
0.1 *M* phosphate	8.0	4.4	80.5	68.0	47.5	13.4
0.1 *M* veronal	8.0	4.3	77.0	65.5	47.6	15.0
0.1 *M* bicarbonate	8.0	4.5	74.6	61.0	47.0	19.0

[a] 8% BSA, 30% glucose.

[b] At 500 mμ; dilution, 1 to 10; read against controls containing no protein and the pH of which did not change.

[c] After 689 hr., the products isolated from the residual solution had analyses as follows: phosphate, 13.1% total nitrogen, 0.22% amino nitrogen; veronal, 13.1% and 0.25%; bicarbonate, 13.1% and 0.28%, respectively, on a dry basis.

Properties of the Serum Albumin-Glucose Product

A number of preparations were isolated after the reaction between BSA and glucose had been allowed to proceed to "completion" under varying conditions of concentration, temperature, pH, *etc.* Their properties were found to be closely similar and the data in Table III are, therefore, representative. After dialysis, the brown solution could be lyophilized to a red-brown powder. Recovery of nitrogen was quantitative, and the yield of product was approximately 120–125% by weight of the BSA used. The product was soluble in dilute alkaline and acid solution but insoluble at its isoelectric region which, as measured by solubility, appeared to be at pH 4.2–4.4 (Table IV). The decrease from that of BSA (pH 4.6) (31) is due both to the loss of total basic groups, attributable to the reaction of glucose with the amino groups, and to an increase in total acid groups, presumably part of the browned carbohydrate fragment.

In addition to its insolubility in the isoelectric range, the derivative differed from BSA in its resistance to coagulation by heat. Solutions of the derivative were heated to 100°C. for several hours both below (pH 3.5) and above (pH 7.0) the isoelectric region, with no trace of precipitation or change in behavior with regard to solubility at the isoelectric point. The development of resistance to denaturation is illustrated in Table VI. In this property the BSA derivative resembles ovomucoid

TABLE III

Comparison of the Properties of Bovine Serum Albumin with Those of Product Obtained by Reaction with Glucose[a]

Properties	BSA	BSA-glucose
Total nitrogen	16.1%	13.1%
Amino nitrogen	1.2%	0.2%
Total basic groups/10^4 g.	15.9	9.2
Total acid groups/10^4 g.	15.8	24
Total reducing groups/10^4 g.	0	8
Isoelectric region	pH 4.6–4.8	pH 4.2–4.4
Solubility at isoelectric point	>25%	0.2%[b]
Color	None	Brown

[a] All analyses are reported on a moisture-free basis.
[b] At pH 4.35. Enhanced somewhat in the presence of salt.

TABLE IV

Solubility of BSA-Glucose Derivative in Solutions of Varying pH

pH	Percentage of total nitrogen in solution[a] *per cent*
3.85	100
3.94	79
4.19	20
4.38	18
4.73	56
4.80	99

[a] 50 mg. of the derivative was dissolved in water, and varying amounts of 0.05 *N* acid were added. The total final volume was 5 ml. At the point of minimum solubility, the derivative was soluble to the extent of 0.16%.

TABLE V

Decrease in Heat-Denaturability During Formation of BSA-Glucose Derivative

Method of preparation-[a] duration of treatment *days*	Amino nitrogen content *per cent*	Time for formation of gel at 100°C.[b] *min.*
0	1.1	1–2
1	0.6	8–10
3	0.36	20–40
5	0.29	No gel[c]
8	0.23	No gel[c]

[a] 8% BSA, 30% glucose, pH 7.0, 53°C.
[b] 100 mg. of BSA or its isolated glucose derivative were dissolved in 2.2 ml. of 0.34 *M* phosphate buffer (pH 8.0) and heated in sealed test tubes in a boiling-water bath.
[c] After 4 hr.

(26% carbohydrate) which does not coagulate on heating, although such treatment does result in the loss of its ability to inhibit trypsin (32).

Electrophoretic homogeneity of BSA and of the BSA-glucose derivative at pH 3.6 was indicated by Tiselius diagrams (Fig. 4). We are indebted to W. H. Ward for the determinations. The electrophoretic mobility of a product of the reaction of BSA and 30% glucose (pH 7, 53°C., 8 days) was compared with that of the untreated protein. The runs were made at pH 3.6–3.7 in a buffer of ionic strength 0.1, containing 0.08 *M* NaCl, 0.02 *M* sodium acetate, and 0.2 *M* acetic acid. The observed mobilities in units of 10^{-5} cm.^{2}/volt-second at 0°C. were: for the BSA-glucose derivative, falling boundary, + 2.53: rising boundary, + 2.71; for BSA, falling boundary, + 4.31; rising boundary, + 4.84. The slower mobility of the derivative was to be expected in view of the decreased amino nitrogen and increased acid group content.

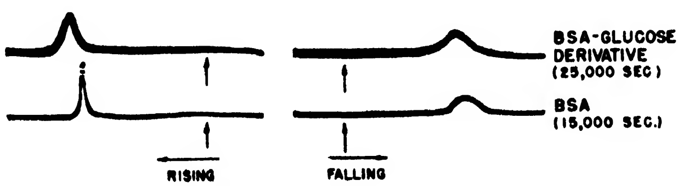


FIG. 4. Tiselius diagrams for BSA and BSA-glucose derivative, pH 3.6–3.7 (see text).

Osmotic pressure measurements were used to estimate molecular weights. The technique used was essentially that of Bull and Currie (33). One preparation, obtained by reaction with 30% glucose for 12 days at 53°C., had an average molecular weight of 291,000. Another, prepared under the same conditions except that the glucose concentration was 50% and the time 14 days, had a molecular weight of 107,000. These values were obtained by extrapolation of 6 separate determinations to 0 concentration. A third preparation was measured (at 1.5% concentration) in buffer-6.7 *M* urea solution to determine whether association was a factor in these results. The osmotic pressure in urea corresponded to an apparent molecular weight of 160,000, compared to 222,000 for a similar preparation run at the same concentration but without urea. Osmotic pressure measurements with untreated BSA (in the presence or absence of urea) gave values of 65,000–69,000, in reasonable accord with the accepted molecular weight of 67,000. As discussed in more

detail below, we interpret the increased molecular weights of the BSA-glucose derivatives as showing some cross-linking, similar to that observed in this laboratory with formaldehyde (34).

A single sample of glucose-treated BSA was examined in an electrically driven ultracentrifuge (Spinco[4]) by W. H. Ward. The diagram obtained at the end of 65 min. at top speed is shown in Fig. 5. The sharp peak on the low-molecular-weight side corresponds roughly in sedimentation rate with that of BSA itself run under the same conditions (4.5 and 4.1, respectively). On the high-molecular side, there is a suggestion of several components with sedimentation rates (6.9 and 8.6) that might be accounted for by cross-linked derivatives containing 2 and 3, respectively, of the original BSA molecules.

FIG. 5. Ultracentrifuge pattern of BSA-glucose derivative (see text).

The absorption spectra of BSA and its brown glucose derivative are shown in Fig. 6. The visible spectrum of the latter is quite without identifying character.[9] The band with maximum at 280 mμ probably is due to the aromatic amino acids of the BSA itself, but combined 5-(hydroxymethyl)furfural, which has a sharp peak in this region (35, 36), or a compound of related composition might be responsible. To determine whether a more distinctive spectrum in the visible region would appear at a different pH, solutions were adjusted to pH 2.3, 6.7, 9.1, and 10.7 and examined in a recording spectrophotometer at 400–740 mμ. Absorption was less in acid and greater in alkaline solution (percentage transmissions at 500 mμ were 33, 28, 23, and 19, respectively), but no bands were detectable.

Inasmuch as the digestibility of the browned protein derivative might throw some light on the nutritive value of foodstuffs that had

[9] The peak in the spectrum of BSA at 400 mμ has not previously been described. It was observed also with a solution of gliadin. The significance of these observations is not known.

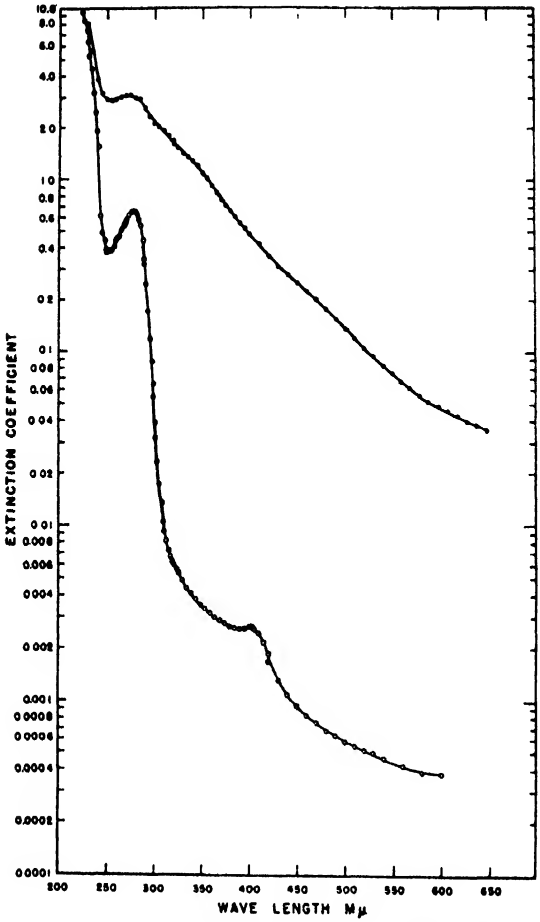


FIG. 6. Ultraviolet absorption spectra of BSA (bottom) and the brown BSA-glucose derivative (top).

deteriorated through the browning reaction, it was of interest to determine the relative rates of digestion of BSA and its glucose derivative by crystalline trypsin. The results of one experiment[10] indicated that

[10] Protein equivalent to 100 mg. of BSA was dissolved in 5 ml. of 0.14 *M* phosphate buffer (pH 7.6), to which was then added 0.1 ml. of 0.01 *N* NaOH and 1 ml. of trypsin solution containing 0.2 mg. crystalline trypsin in 0.001 *N* HCl. At the end of 16 hr. incubation at 35°C., 4 ml. of formalin and 1 g. of solid urea were added. The solutions were titrated to pH 8.5 with 0.02 *N* NaOH. The differences between the titration data obtained at 0 time and after 16 hr. were, for BSA, 1.34 ml., for BSA-glucose derivative, 0.76 ml., for BSA-glucose derivative previously heated in solution for 4 hr. at 100°C., 0.34 ml.

the derivative is attacked by trypsin but at a rate lower than that demonstrable with native BSA. Heat treatment of the glucose derivative (solution heated 4 hr. at 100°C.) decreased the digestibility still further.

Browning of Heat-Denatured BSA

It was considered of interest to determine whether denaturation might affect the course of the BSA-glucose reaction. Denatured BSA was prepared as follows: A 10% solution of BSA in distilled water was heated for 20 min. at 100°C. The gelled mass was then dried by lyophilization and allowed to come to equilibrium with atmospheric conditions for analysis. A suspension of this preparation was heated at 53°C. with 30% glucose solution at pH 7.0 for 5 days, then dialyzed and dried. The resultant deep brown powder contained 14.1% total nitrogen and 0.23% amino nitrogen (dry basis), compared to 16.1% total nitrogen and 1.15% amino nitrogen in the denatured BSA, preparation (*cf.* Table IV). Thus, except for being in suspension rather than in solution, there was no apparent difference in the reaction of denatured BSA, as compared with native BSA, with glucose.

Scatchard *et al.* (37) showed that measurable denaturation could be detected in 25% BSA solutions within the range of pH 6.9–7.4 at 54.7°C. in 2–4 days. However, it is probable that such changes could not have been important in the reaction of native BSA with glucose, first, because the more dilute solutions are more stable, second, because of the stabilizing effect of high sugar concentrations, and third, because the rates of browning within the range of 25–65°C. are in agreement with kinetic theory (Fig. 2).

Involvement of Protein Groups

The rate of loss of detectable amino groups during the reaction of BSA with glucose is shown in Table VI. Changes in the amino nitrogen contents of other proteins and model substances are given in Table VII.

That the "browning" depends upon the presence of free amino groups at temperatures of 53°C. or below was shown with acetylated and guanidylated serum albumin derivatives, in which the amino but, presumably, no other group had been changed. These products did not brown appreciably even after two weeks at 53°C. in 30% glucose solution. After 10 days, one preparation of acetylated BSA was recovered by dialysis and found to have the same nitrogen content (15.5%) as that of the protein prior to exposure to the glucose treatment. Thus, at 53°C. or lower, a protein lacking appreciable amounts of free amino groups does not brown or undergo other types of reaction with glucose. However, at 70°C., acetylated BSA did turn brown under the condition used[11] and evidence was obtained that other groups, possibly guanidyl groups, had reacted (Table VIII).

[11] The solution of acetylated BSA in 30% glucose (pH 7.0) gelled within two hours at 70°C.

That guanidyl groups do not participate *directly* in the browning reaction at a temperature of 53°C. or below was suggested by the fact that several model substances containing a considerable number of these groups (edestin, guanidylated human serum albumin, and acetylated

TABLE VI

Comparison of Rate of Browning of BSA-Glucose Solutions[a] With Loss in Amino Nitrogen and Total Nitrogen, Apparent Bound Glucose, and Formaldehyde Yield after Periodate Oxidation

Time	pH	Transmission	Amino nitrogen[b]	Total nitrogen	Bound carbohydrate[c]	Apparent bound glucose[d]	Formaldehyde yield[e]
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
2 hours	6.9	97	1.2	15.8	1.6	0.1	0.07
2 days	6.9	73	0.4	14.7	8.8	0.9	0.37
6 days	6.8	26	0.3	14.1	12.0	1.8	0.53
7 days	6.7	16	0.2	13.8	14.3	1.9	1.13
13 days	6.6	9	0.1	13.6	15.8	2.4	1.11
15 days	6.3	5	0.1	13.3	17.3	2.6	1.08

[a] 8% BSA, 30% glucose, pH 7.0, at 53°C. All analyses on dry basis.
[b] Van Slyke method, 15 min. (18).
[c] Calculated from the decrease in total nitrogen (BSA, 16.1%).
[d] Orcinol procedure (24).
[e] After periodate oxidation (28).

TABLE VII

Effect of Reaction with Glucose on Amino Nitrogen Content of Proteins and Model Substances[a]

	Amino nitrogen Original	Amino nitrogen Glucose-treated[b]
	per cent	*per cent*
Bovine serum albumin	1.2	0.2
Lysozyme	0.8	0.2
Chymotrypsinogen	0.8	0.3
Polyamino compound[c]	4.6	0.7
Polyamino compound[d]	3.9	0.5

[a] Heated at 53° for 5 days in a solution containing 30% glucose and buffered (0.56 *M* phosphate) at approximately pH 7.0.

[b] These values are probably too high because of the reducing capacity of the browned derivative (footnote 5).

[c] Prepared by polymerization of tyrosine with formaldehyde. Total nitrogen, original, 6.6 per cent; glucose-treated, 4.1 per cent.

[d] Prepared by reaction of hexamethylene diamine with polyglutamic acid methylester (see Preparative). Total nitrogen, original, 15.7 per cent; glucose-treated, 11.3 per cent.

bovine serum albumin) did not turn brown during exposure to the buffered glucose solution. Additional evidence was obtained by quantitative analysis for guanidyl groups (Table VIII). Also, solutions of methyl guanidine sulfate in 30% glucose could be heated at 100°C. for several hours at pH 7 without developing color. Nevertheless, the apparent arginine contents of both BSA and protamine sulfate were decreased after the reaction with glucose. Previous work with formaldehyde (38)

TABLE VIII

Effect of Reaction with Glucose on the Apparent Guanidyl Content of Proteins

Protein	Apparent arginine nitrogen as per cent of total nitrogen	
	Before reaction *per cent*	After reaction[a] *per cent*
At 53°C., 6 days		
Guanidylated human serum albumin	33.7	33.8
Edestin	27.8	28.8
Acetylated bovine serum albumin	11.3	11.5
Bovine serum albumin	11.8	7.2
Protamine sulfate	84.6	74.9[b]
Ovomucoid	8.6	6.5[c]
Edestin and alanine[d]	27.8	23.7
At 70°C., 4 days		
Guanidylated human serum albumin	33.7	26.3
Edestin	27.8	21.3

[a] Unless otherwise indicated, 1 g. protein, 10 ml. of 30% glucose, and 2 ml. of 3.4 *M* phosphate buffer (pH 8.0) were brought to a total volume of 12.5 ml. The mixtures were at pH approximately 7.0. They were heated in an air oven at the temperatures and for the times indicated, isolated by dialysis, and dried by lyophilization.

[b] By analysis of a product obtained after 6 hr. of dialysis. The very small yield of material (10%) obtained by prolonged dialysis of the reaction mixture had 61.9% of its nitrogen as arginine nitrogen.

[c] After 3 days at 53°C. After 19 days at 53°C. the arginine nitrogen value was 4.1%.

[d] Alanine was present in amount corresponding to 20% of the weight of the protein.

suggested that the guanidyl groups might be involved in a type of cross-linking reaction requiring the presence of a free amino group. In conformity with this hypothesis it was found that the apparent arginine content of edestin was decreased when the glucose reaction was carried out in the presence of alanine (Table X). The edestin-alanine-glucose-buffer system became very brown, as did also the control containing no edestin, but in both cases most of the color was removed by dialysis. The results indicate that guanidyl groups are involved in sec-

ondary reactions only when amino groups are available to initiate the primary reaction.

Work with formaldehyde had shown that methylene cross-linking could occur between amino and guanidyl groups or amino and amide groups (38). Hence, it appeared of interest to determine whether amide groups also might be involved in secondary reactions with glucose. Polyglutamine was used as a model substance. The material (8%) was heated for 5 days at 53°C. in 30% glucose solution buffered at pH 7, with and without added alanine (1.1%). Only when alanine was present did the solution turn brown. Both solutions were dialyzed, during which the brown color of the one was lost, and the polyglutamine was recovered by lyophilization. The products contained 18.2% and 18.9% total nitrogen, compared to 18.5% total nitrogen in the original prepa-

TABLE IX

Microbiological Amino Acid Analyses of Bovine Serum Albumin and its Glucose Derivative[a]

Amino Acid	Bovine serum albumin	Glucose derivative[b]	Difference
	per cent	*per cent*	*per cent*
Arginine[c]	5.8	2.6	−55
Aspartic acid	10.2	10.5	+3
Glutamic acid	16.2	16.0	−1
Glycine	1.7	1.7	0
Histidine	3.7	3.0	−19
Isoleucine	3.7	3.7	0
Leucine	12.0	12.3	+2
Lysine	11.5	4.9	−57
Methionine	0.8	0.7	−12
Phenylalanine	6.3	6.0	−5
Proline	4.7	5.1	+8
Threonine	7.6	7.8	+3
Tyrosine	4.9	4.1	−16
Valine	8.3	8.3	0

[a] The two proteins were acid-hydrolyzed and assayed microbiologically under identical conditions. We are indebted to Neva Snell and J. C. Lewis for the data. The procedures used will be described elsewhere by them.

[b] Contained 13.2% nitrogen, dry basis. Results are expressed in terms of the BSA present.

[c] Arginine values by colorimetric analysis (21) were 5.9% and 3.6%, respectively (*cf.* Table VIII).

rations (values uncorrected for moisture). It may be concluded that amide groups do not appear to be involved either directly or indirectly in the reaction with glucose under the conditions used.

Reaction of amino and guanidyl groups was also indicated by microbiological assays of acid hydrolyzates of BSA and its glucose derivative (Table IX). Contents of both lysine and arginine were found to be decreased by more than 50%. The other amino acids assayed, with the possible exceptions of histidine and tyrosine, appeared not to have been appreciably affected.[12] In this connection, it is of interest that Patton, Hill and Foreman (39) found that the essential amino acids apparently destroyed in part by *refluxing* casein with 5% glucose

TABLE X

Effect of Glucose Reaction on Tryptophan Content of Proteins[a]

Protein	Reaction time	Total nitrogen	Amino nitrogen	Apparent tryptophan content[b]
	days	*per cent*	*per cent*	*per cent*
BSA	0	16.1	1.2	0.8
BSA	5	13.9	0.3	0.0
Lysozyme	0	18.0	0.8	8.0
Lysozyme	2	16.9	0.3	6.5
Lysozyme	5	16.4	0.2	3.9
Chymotrypsinogen	0	16.4	0.8	6.0
Chymotrypsinogen	5	14.3	0.3	3.6

[a] 53°C., 30% glucose, 8% protein, 55 *M* phosphate, final pH about 7.

[b] Analyzed without hydrolysis by the colorimetric method of Horn and Jones (20). Approximate values only.

solution for 24 hr. were lysine, arginine and tryptophan, and that these, together with histidine, were involved in a soybean globulin-glucose product prepared similarly. Work in this laboratory with formaldehyde had shown that amino groups could be cross-linked with phenol, imidazole, and indole groups under somewhat more drastic conditions than those required for the involvement of amide or guanidyl groups (40). Patton *et al.* (39) did not assay for tyrosine.

Tryptophan analyses (Table X) indicated that indole groups were affected in the reaction with glucose. Where these were scarce, as in

[12] Partial loss of some of the amino acids might have occurred in the course of the acid hydrolysis.

BSA, almost all the tryptophan had reacted. In the case of lysozyme, most of the indole groups were still present. Possibly the involvement of indole residues is also a secondary reaction similar to that of the guanidyl groups (*cf.* 40). When gramicidin, a polypeptide containing many indole groups but no amino groups, was treated with 30% glucose solution at 53°C., there was no browning, and biological assays also indicated that no reaction had occurred (41).

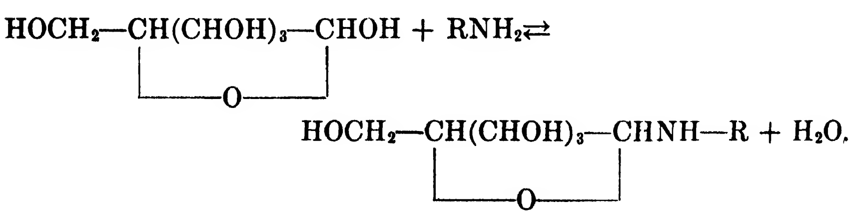
Preparative

Polyamine Compound B

One g. of polyglutamic acid methyl ester (42) was treated with 9 ml. of hexamethylene diamine at 40°C. for 3 days. The resultant homogeneous gel was dialyzed for 3 days against running tap water and then, in turn, against distilled water, dilute acetic acid, dilute HCl, and distilled water. It remained a soft gel, filling the dialysis bag. Approximate yield, after lyophilization, 1.6 g. Anal.: Calc'd for ($C_{11}H_{21}O_2N_3 \cdot HCl$): N, 15.9; amino N, 5.3; basic groups, $38/10^4$ g.; Found (dry basis): N, 15.7, amino N, 4.5; basic groups, $33/10^4$ g.

Discussion

While the reactions occurring in solutions of protein and glucose are not known with certainty, a brief discussion of some of the possibilities is warranted. It appears likely that the first reaction involves the addition of glucose to the amino groups of the proteins, and is reversible. Przylecki (43) described such protein-carbohydrate "symplexes." The reaction would be analogous to (a) the addition of formaldehyde to amino groups to form mono- or dimethylol compounds or (b) to the addition of glucose to simple amines to form N-glycosides. Some examples of the latter reaction have been described by Mitts and Hixon (44). The products are white crystalline compounds which hydrolyze when dissolved in water.



The induction period phenomenon (Fig. 1) is possibly associated with this stage. Frankel and Katchalsky (45,46) showed that there was a

definite time lag in the reaction of glucose with amino acids and peptides, as studied by changes in pH of aqueous solutions.

The second stage of the reaction results from a decomposition of the first reversible complex and is accompanied by the development of color. The primary amino groups can no longer be completely recovered by hydrolysis.[13] The carbohydrate fragment also is irreversibly bound. Presumably, it is the decomposition of the carbohydrate part of the complex that gives the brown color. It has been supposed that this color may be due to a polymerization product of the carbohydrate

TABLE XI

Effect of Acid Hydrolysis on BSA-Glucose Derivative at 100°C.[a]

Reagent[b]	Time *hr.*	Glucose *per cent*
0.1 *N* HCl	3	0.4
1 *N* HCl	4	3.1
1 *N* HCl	16	3.1
1 *N* HCl	30	4.1

[a] 100 mg. of the derivative was heated with 5 ml. of acid. After the time periods indicated, 5 ml. of NaOH of the same concentration was added, and the solution was analyzed for glucose after deproteinization by Somogyi's method (23). No detectable glucose was released in 24–72 hr. in 0.1 *N* NaOH or 0.1 *N* HCl at room temperature. By the orcinol procedure (24) the product used appeared to contain 1.6–1.7% bound glucose.

[b] Only 0.2% glucose was found after hydrolysis with 6 *N* HCl for 24 hours but glucose itself was 95% destroyed by these conditions.

fragment (*cf.* 47), but such interpretation is not supported by the present studies, since the average molecular weight of addition product per amino group lost (150–250) was approximately equal to that of a glucose molecule (180)[14]. The work of Lea (11) with dried-milk protein also indicates that the amount of addition of carbohydrate to the amino group is roughly in the molecular proportion of 1:1.

[13] The possibility of freeing amino groups could best be investigated with the tyrosine-formaldehyde polymer. The original polymer contained 5.0% amino nitrogen, unchanged after hydrolysis with 6 *N* HCl under reflux for 18 hr. A glucose derivative contained 1.85% amino nitrogen before, but 2.5% amino nitrogen after, a similar hydrolytic procedure. A small but measureable part of the bound glucose could be split off by mild acid hydrolysis (Table XI).

[14] Estimated from decreases in total nitrogen compared to decrease in amino nitrogen (Tables VI, VII, X). Such calculations suffer from the inaccuracy of the amino nitrogen determination (footnote 5).

TABLE XII

Comparison of the Amount of Color in a Browned BSA-Glucose Solution[a] *with that Accountable for by the Isolated Protein Derivative*

Time	pH	Percentage transmission		Nitrogen content[c]
		Before dialysis	After isolation[b]	
days		*per cent*	*per cent*	*per cent*
1	7.4	85.5	84.6	13.6
3	7.3	43.0	42.3	12.9
5	7.1	21.6	21.5	12.7
8	7.1	11.0	10.5	12.3

[a] 8% BSA, 30% glucose, 0.55 *M* phosphate, 53°C.

[b] The samples were dialyzed for 7 days, lyophilized and then dissolved in a solution containing glucose and phosphate buffer to the same concentration used in the storage experiment. The pH also was adjusted to that of the original solution. The color was measured against a similar solution but containing no protein.

[c] Of the dried product; not corrected for moisture.

The nature of the brown fragment has eluded identification, although several groups of investigators have been actively engaged in the study. Singh and coworkers (35), among others, studied the browning of glucose solutions at 100°C. and concluded that hydroxymethylfurfural is an important intermediate. It appears probable, however, that the reactions at 100°C. differ from those described in this paper. Enders (48) considered methylglyoxal to be the chief intermediate in "humin" formation. Haas *et al.* (49) found that the browning of apricot syrup (pH 4) was inhibited by continuous extraction with ethyl acetate, indicating that important intermediates were being removed. However, continuous extraction of a BSA-glucose solution either at pH 4 or 7 with ethyl acetate did not affect the rate of color formation. If either methylglyoxal or hydroxymethylfurfural were intermediates, some alteration in rate might have been expected.

A detailed discussion of possible structures is not warranted at this time. However, the following points can be made. Many of the carbohydrate fragments have measureable reducing capacity[15] and contain

[15] The number of reducing groups/10^4 g. of original BSA after 1, 3, 5, and 8 days of reaction with 30% glucose at 53°C. were approximately 1.5, 3, 5, and 8, respectively, as determined by the ferricyanide method devised by Mirsky and Anson (25) for cysteine. If the structure responsible for the brown color were unsaturated, it should be possible to effect some change by hydrogenation. However, two attempts to hydrogenate a browned tyrosine-formaldehyde polymer in aqueous-alcohol solution with platinum catalyst were unsuccessful. No hydrogen was absorbed.

groups of an acidic nature (Table III), and some yield formaldehyde with periodate (Table VI). The protein derivative contains *all* of the color of the solution (Table XII), which suggests that the protein does not catalyze the browning of carbohydrate not directly attached to it, as has been suggested at times (47), but instead is an integral part of the reaction.

It has recently been shown in this laboratory that proteins "brown" with acetaldehyde and propionaldehyde even more readily than with glucose.[16] It is thus apparent that browning is not limited to reactions with reducing sugars or their derivatives, but that the aldehyde grouping is essential.[17]

Acknowledgments

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Summary

The reaction of proteins, protein derivatives, and model substances with glucose solutions has been studied in detail. The rate of browning is proportional to the temperature and pH. The free amino groups are the primary site of the reaction. They decrease as the reaction proceeds, and if they are masked, as by acetylation, no browning occurs at or below 53°C. Guanidyl groups are involved secondarily, but not amide groups. Tryptophan, and possibly histidine and tyrosine, is also affected.

The browned derivative of bovine serum albumin has been characterized in detail by electrophoretic, ultracentrifuge, osmotic-pressure, amino acid analyses, and other techniques.

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Biochemical Individuality. IV. A Paper Chromatographic Technique for Determining Excretion of Amino Acids in the Presence of Interfering Substances

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Introduction

In the study of biochemical individuality (1) previous work suggested the existence of individual patterns of amino acid excretion (2). There was need for a routine procedure which might be applied to determine the amino acids present in urine samples collected from a large group of individuals over a long period. The use of paper chromatography, as modified by Williams and Kirby (3), makes possible the rapid analysis of large numbers of samples. While a paper chromatographic technique does not possess the accuracy of microbiological or chemical methods in determining amino acids, it is a potentially valuable tool in revealing relatively large differences in individual excretion patterns.

In attempting to adapt the method suggested by Polson (4,5) to the determination of amino acids in urine, which contains relatively large quantities of salts and other constituents, early experiments showed that amino acids added to urine do not migrate to the same position as the amino acids in a salt-free mixture (see Fig. 1). For an extensive survey of large numbers of urine samples, it seemed desirable to develop a procedure which would eliminate, as far as possible, time-consuming purification of the sample through use of electrolytic "desalting" apparatus (6), or removal of urea by enzymatic hydrolysis. In the analysis for a specific amino acid in urine, the superposition of a known amount of the amino acid to a spot to which urine has been applied and allowed to dry reproduces the same conditions of migration in the pres-

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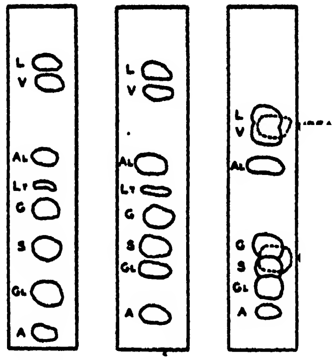

A. B. C.

FIG. 1.

A. Typical chromatogram of salt-free mixture of amino acids

B. Typical chromatogram of amino acids in urine of average salt content

C. Typical chromatogram of amino acids in concentrated urine[a]

[a] Neither chloride nor urea give a visible reaction with ninhydrin. Identical sheets have been developed with suitable reagents for the detection of chloride and urea. The dotted areas show their respective positions.

L—leucine; V—valine; Al—alanine; Ly—lysine; G—glycine; S—serine; Gl—glutamic; A—aspartic. (Phenol used as solvent.)

ence of the constituents of the particular urine for the known amount of amino acid as for the unknown amount of amino acid in the sample. This makes possible a practical approach to the determination of amino acids in urine as described below.

Experimental Procedure

1. *Application of Sample*

For the analysis of one sample of urine, the long edge of a sheet of Whatman No. 1 filter paper (28 × 45 cm.) is marked with a series of small dots 2 cm. apart and 2.5 cm. from the edge which is to be inserted in the solvent. This gives 20 positions for application of the urine sample and the dilutions of the standard amino acid solutions. Light pencil marking of the spot causes no interference and greatly facilitates the preparation of the sheet.

Beginning at the left bottom edge, urine is added to the pencilled dots as shown in Table I. The solution is applied using a 5 µl. capillary pipette (Microchemical Specialties Co., Berkeley, Calif.). For amounts larger than 5 µl., a second application is made after the first spot is thoroughly dried, a third in the same manner, and so on, until the desired volume on the single spot has been added. The use of 5 µl. increments permits the size of each spot to remain uniform. Of the animo acids which are normally present in detectable amounts in urine, those which may be separated using phenol as

a solvent were selected for this study. This group includes aspartic acid, glutamic acid, serine, glycine, alanine, valine, and leucine-isoleucine (listed in the order of increasing R_f values). Leucine and isoleucine go to the same position. Leucine was used as the standard.

These amino acids, which may be resolved by phenol, are divided into two groups and stock solutions prepared as follows:

Solution I—1 mg./ml. each of aspartic acid and serine.
2 mg./ml. of leucine.
Solution II—1 mg./ml. each of glutamic acid, glycine and alanine.
2 mg./ml. of valine.

Solutions I and II are then prepared in 1:5, 1:6.67, 1:10, and 1:20 dilutions and applied to the urine spots as indicated in Table I. Thus, on the first spot there will be 5 μl. of urine to which has been added 1 γ each of aspartic acid and serine and 2 γ of leucine; the second spot contains the same concentrations of the above amino acids together with 10 μl. of urine; the third spot contains 5 μl. of urine to which has been added 1 γ each of glutamic acid, glycine and alanine, and 2 γ of valine; *etc.*

TABLE I

Spot positions (2 cm. apart)	Urine added	Dilutions used (5 μl. of each dilution added)	
		Solution I (aspartic acid, serine, and leucine)	Solution II (glutamic acid, glycine, alanine, and valine)
	μl.		
1	5	1:5	
2	10	1:5	
3	5		1:5
4	10		1:5
5	5	1:6.67	
6	10	1:6.67	
7	5		1:6.67
8	10		1:6.67
9	5	1:10	
10	10	1:10	
11	5		1:10
12	10		1:10
13	5	1:20	
14	10	1:20	
15	5		1:20
16	10		1:20
17	5	1:20	
18	15	1:20	
19	5		1:20
20	15		1:20

For purposes of comparison, the spots which contain added amino acids in a small volume of urine are adjacent to those derived from a larger volume of urine with no added amino acids. The range utilized here is 0.20 mg./ml. as the highest concentration of amino acid (0.40 mg./ml. of leucine and valine) and 0.025 mg./ml. as the lowest concentration which may be determined on one sheet. The amino acids in most urine samples will fall within this range. However, by running chromatograms of diluted urine, the upper limit may be raised, and by use of larger volumes of the urine sample, the limit may be extended downward if necessary. A longer time is required for the application of the sample when larger volumes are used, since each increment of 5 µl. must be dried completely before the next is applied. The accuracy is increased by using a greater number of dilutions.

2. Solvent

Phenol has been used most extensively in the present investigation. To counteract the effect of inorganic salts in the urine which produce a greater diffusion of amino acids bands, salts were added to the solvent mixture. Sodium citrate and KH_2PO_4 were chosen since they are also effective in inhibiting the migration of a contaminant in the paper which damages the lower half of a chromatogram when water-saturated phenol is used. Ascorbic acid was found to prevent the discoloraion of the phenol solution. The solvent is prepared by mixing 100 g. of phenol (analytical grade) with 20 ml. of a solution which contains 6.3% sodium citrate, 3.7% KH_2PO_4, and 0.5% ascorbic acid, and allowing this mixture to stand (with occasional stirring) until solution occurs. The upper layer is decanted and used. The solvent mixture may be used over again but should not be kept more than 48-72 hr.

3. Capillary Ascent Technique

After each spot has been prepared, the sheet is fastened as a cylinder with staples so that the edges do not quite meet, and is then placed upright in a shallow Pyrex dish containing the solvent mixture. The dish and cylinder are placed in a 6-gallon stone jar fitted with an air-tight lid (3). Twelve to sixteen hr. are required for the solvent to ascend.

4. Drying

One of the most critical points in the preparation of a paper chromatogram involves removal of the solvent. Amino acids are relatively easily oxidized, and prolonged heating renders them unreactive to ninhydrin. The intensity of color which develops from the same amount of amino acids was found to decrease either with an increase in temperature over 80°C. or with an increase in the time of heating at the same temperature. Phenol is not easily removed from a sheet by allowing it to stand at room temperature. The use of the lowest temperature which will remove the solvent in a reasonable time seems most desirable and, for consistent results, heating should be uniform. Removal of the solvent was found to be accomplished best by blowing heated air at 85°C. over the sheets for 8–10 min.

5. Preparation of a Two-Dimensional Chromatogram

It cannot be assumed that a spot in the position of alanine, for example, on a chromatogram run in phenol is due solely to that amino acid unless further identification

procedures are used. To eliminate the possible interference caused by other ninhydrin-reacting substances, whose positions may coincide with certain of the amino acids being determined, it is necessary to run a two-dimensional chromatogram to detect their presence. Glutamine, β-alanine, citrulline, and tyrosine, if present, exhibit about the same R_f values as alanine. Asparagine, taurine, and glycine cannot be distinguished from each other on a one-dimensional chromatogram using phenol as the solvent.

A two-dimensional chromatogram for this purpose is prepared by adding 25 μl. of the urine to a spot 3 cm. from the corner of a sheet 14 × 22.5 cm. This is formed into a cylinder and, for the first run, is set inside the larger 20-spot cylinder and run simultaneously with it. After drying, this inner cylinder is unfastened and re-formed so that the second run can be made at right angles to the first. The second run is made using collidine as solvent. The composition of the collidine-water mixture depends on the purity of the collidine (7).

The positions on such a two-dimensional chromatogram of the amino acids which may be determined using the procedure described are quite characteristic. However, if the chromatogram of 25 μl. of urine reveals the presence of a substance which overlaps the position of one of the amino acids when phenol is used as a solvent, no attempt is made to determine that amino acid. In our experience, as little as 0.2 γ of amino acid can be detected on chromatograms prepared as described. If taurine, for example, were present in amounts too small to be detected in 25 μl. of urine, the concentration must be less than 0.008 mg./ml. The range of concentration of amino acid which can be determined by this procedure is from 0.025 to 0.20 mg./ml. If taurine were present at a concentration of 0.008 mg./ml., the resulting error in the glycine determination at the highest value of 0.2 mg./ml. of glycine would be 4%. At a glycine concentration of 0.025 mg./ml., the error due to the presence of taurine would be 32%.

The two-dimensional sheet provides additional qualitative information regarding the amino acids present in the urine sample. Amino acids other than the 8 mentioned in the earlier part of the paper may be determined provided the composition of the urine is such that their positions do not overlap. For example, in our experiments, when threonine and arginine were absent as indicated by the two-dimensional chromatogram, lysine was included in the group to be determined.

6. Development with Ninhydrin

Using 0.1% ninhydrin in butanol, the lowest concentration of amino acid which can be detected is 0.5 γ. A solution of 0.2% ninhydrin in butanol saturated with water when used to spray the dried sheets in the usual way, increases the sensitivity of the color reaction of the amino acids so that as little as 0.2 γ of glycine, serine, and alanine can be detected. Ninhydrin (0.2%) in water saturated butanol (6) gives a much more intense color for the same amount of amino acid than does 0.2% ninhydrin in butanol alone. Ninhydrin dissolved in a mixture of equal parts of butanol and ethanol produces an even more intense color for the same concentration of amino acid, but the color fades more rapidly. Heating for 5 min. at 100°C. is sufficient to bring out the color. Heating the sheet after spraying with ninhydrin is not so critical as heating during removal of the solvent. The extent of the visible reaction of amino acids with ninhydrin does not increase appreciably with increase in temperature or in time of heating.

7. *Matching*

Matching of colors of a particular amino acid after the development process is completed involves the judging of spots which are in close proximity to one another. Thus, the unknown amount of urinary glutamic acid in 10 μl. of urine (Position 2 in Table I) may be compared for intensity of color to the glutamic acid in 5 μl. of urine plus 1 γ of added glutamic acid (Position 3). Placing the spots side by side makes comparison of colors more exact. It the amounts of glutamic acid in these two positions seem to be the same, then, by difference, the glutamic acid present in 5 μl. of urine is estimated to be 1 γ, since the amount in 10 μl. of urine is equal to 1 γ of glutamic acid plus the amount present in 5 μl. of urine.

Analytical Results

The results of urine analyses, including recovery experiments in which unknown additions were made by an outside party, are presented in Table II. In this case, matching was done independently by 3 observers, one of whom had no experience with the method other than reading the present discussion. The values recorded are averages of the results obtained from duplicate chromatograms. When the amino acids were found to be present in concentrations greater than 0.2 mg./ml., chromatograms were run a second time using diluted urine. In our experience, barring mistakes of a clerical nature involving comparison of the wrong spots, various observers consistently agree on each individual value within a range of 15%, and the values obtained from duplicate chromatograms agree with each other with an average deviation of about 5%. The errors inherent in the method as applied to urine are largely due to the interfering substances present.

The recoveries in Table II vary from 63 to 160%. Assuming that a value for urinary amino acid may be as much as 50% in error, differences in individual excretion patterns may still be revealed by this technique, as shown in Table III. Morning urine samples were collected and analyzed daily from a group of subjects for a period of 5 weeks. The values shown for each weekly period are averages of the daily excretion of glycine, serine and alanine. Complete results and statistical treatment will be presented later.

These data indicate that there may be fairly large differences in excretion patterns which are characteristic of the individual, differences which are much greater than the error expected of the method. Subject 1 shows consistently much higher values for excretion of glycine, serine, and alanine than any of the other subjects. For example, the average glycine excretion of Subject 1 for the 5-week period during which the

4 subjects were studied is 200% greater than that of Subject 2, 750% greater than Subject 3, 500% greater than Subject 4. Individual variations in excretion of alanine and serine are marked, though not so pronounced as differences shown in excretion of glycine. The consis-

TABLE II

Urinary Amino Acids Found[b]

Sample no. 1	Glutamic	Aspartic	Serine	Glycine	Lysine	Alanine	Valine	Leucine
Original sample (mg./ml.)	0.0	0.0	0.05	0.15	0.10	0.072	0.0	0.0
After addition (calc. to orig. vol.)	0.16	0.11	0.25	0.26	0.125	0.069	0.0	0.0
Amino acids recovered	0.16	0.11	0.20	0.11	0.025	0.003	0.0	0.0
Amino acids added	0.10	0.075	0.20	0.12	0.0	0.0	0.0	0.0
Error (mg./ml.)	+0.06	+0.035	0.0	−0.01	+0.025	−0.003	0.0	0.0
Per cent recovery of total amino acid present	160	147	100	92	125	96	100[a]	100[a]
Sample no. 2								
Original sample (mg./ml.)	0.0	0.0	.031	.067	0.0	.029	0.0	0.0
After addition (calc. to orig. vol.)	0.0	0.0	.036	.063	.095	.24	.19	.125
Amino acids recovered	0.0	0.0	.005	.004	.095	.21	.19	.125
Amino acids added	0.0	0.0	0.0	0.0	.15	.20	.20	.15
Error (mg./ml.)	0.0	0.0	+.005	−.004	−.055	+.01	−.01	−.025
Per cent recovery of total amino acid present	100[a]	100[a]	115	94	63	105	95	84
Sample no. 3								
Original sample (mg./ml.)	0.0	0.0	.21	.39	0.0	.14	0.0	0.0
After addition (calc. to orig. vol.)	0.0	0.0	.37	.64	.10	.30	0.0	0.0
Amino acids recovered	0.0	0.0	.16	.25	.10	.16	0.0	0.0
Amino acids added	0.0	0.0	.15	.18	.10	.15	0.0	0.0
Error (mg./ml.)	0.0	0.0	+.01	+.07	0.0	+.01	0.0	0.0
Per cent recovery of total amino acid present	100[a]	100[a]	107	139	100	107	100[a]	100[a]
Sample no. 4								
Original sample (mg./ml.)	0.0	0.0	.037	.035	0.0	.039	0.0	0.0
After addition (calc. to orig. vol.)	.15	.125	.031	.031	0.0	.062	.25	.14
Amino acids recovered	.15	.125	.006	.004	0.0	.023	.25	.14
Amino acids added	.15	.10	0.0	0.0	0.0	0.0	.30	.20
Error (mg./ml.)	0.0	+.025	−.006	−.004	0.0	+.023	−.05	−.06
Per cent recovery of total amino acid present	100	125	84	89	100[a]	146	84	70

[a] No amino acids were found in urine before or after additions.

[b] Phenol used as solvent.

TABLE III

Urinary Amino Acids[a]

	Glycine (mg./ml.)			
	Subject 1	Subject 2	Subject 3	Subject 4
First week	0.26	0.11	0.05	0.06
Second week	.34	.14	.02	.10
Third week	.26	.18	.03	.03
Fourth week	.31	.22	.06	.07
Fifth week	.31	.09	.04	.06
Average	.30	.15	.04	.06
	Serine (mg./ml.)			
	Subject 1	Subject 2	Subject 3	Subject 4
First week	0.19	0.03	0.05	0.08
Second week	.23	.06	.02	.10
Third week	.20	.075	.04	.10
Fourth week	.19	.09	.06	.06
Fifth week	.21	.04	.03	.07
Average	.20	.06	.04	.08
	Alanine (mg./ml.)			
	Subject 1	Subject 2	Subject 3	Subject 4
First week	0.20	0.06	0.05	0.07
Second week	.20	.08	.02	.11
Third week	.20	.09	.04	.11
Fourth week	.18	.12	.06	.15
Fifth week	.21	.04	.03	.12
Average	.20	.08	.04	.11

[a] Phenol used as solvent.

tency of excretion of certain amino acids by the same subject from week to week lead us to believe that the error in the determination may be much less than that indicated by recovery experiments.

Analysis of Lactoglobulin

While the method has been developed primarily for determining the amino acids in urine where interfering substances are present, its

application to the determination of amino acids in a protein hydrolyzate is of interest. For a trial analysis on a material of known composition lactoglobulin was used, since Brand *et al.* (8) have presented a complete analysis of this crystalline homogeneous protein. Samples of 10, 13, and 21 mg.[2] were each hydrolyzed with 2.0 ml. of 3 N HCl in sealed flasks at 100°C. for 24 hr. The HCl was removed under reduced pressure, water was added, and the sample evaporated to dryness. The hydrolyzate was not neutralized. The residue in each case was diluted with 20.0 ml. of water. To decide which amino acids could readily be determined, a two-dimensional chromatogram was run, using phenol as the first solvent and collidine as the second solvent. It was found

TABLE IV

Amino Acids in Lactoglobulin

Amino acid	Sample I (13 mg. protein)	Sample II (10 mg. protein)	Sample III (21 mg. protein)	Values from literature
	per cent	*per cent*	*per cent*	
Aspartic acid	11.5	—	11.6	11.4 (8) 11.5 (9)
Glutamic acid	—	20.0	19.3	19.5 (8) 19.1 (9)
Lysine	11.0	11.5	11.3	11.4 (8) 12.6 (9)
Alanine	6.5	—	6.3	6.2 (8); 7.1 (9) 6.2 (10); 7.0 (11)
Leucine and isoleucine	20.9	25.0	24.6	24.0 (8) 21.4 (9)

that, at this dilution of the hydrolyzates of the 13 and 21 mg. samples, aspartic acid, glutamic acid, lysine, alanine, and leucine-isoleucine were visible. In the hydrolyzate of the 10 mg. sample, however, only glutamic acid, lysine and leucine-isoleucine were visible. There were no other amino acids whose positions coincided with any one of these when phenol was used.

The results obtained using paper chromatography are compared in Table IV with values cited by Brand and others (9,10,11). Each value obtained by the paper chromatographic method represents an average of estimates obtained when the sheets were compared for color intensity

[2] Kindly furnished to Dr. R. J. Williams by the late Dr. Max Bergman (1944).

by two people. The sheets were prepared in duplicate, and a wide enough range of the dilute protein hydrolyzates and added amino acids were used so that several combinations which matched closely might be found. Glutamic acid was not determined in Sample I because of transformation of the glutamic acid into pyrrolidonecarboxylic acid (12), which does not react with ninhydrin, during removal of HCl from the hydrolyzate.

Discussion

The experiments reported here are primarily exploratory in nature. Little known as yet about so-called normal metabolic patterns. The method described adequately fulfills our need for a routine procedure which may be applied to large groups of individuals in a short time to determine differences or similarities in amino acid excretion. It is apparent from these results that more accurate analyses can be made in the absence of interfering substances such as are present in urine. Even in the presence of interfering substances, however, the accuracy is sufficient for many purposes. We are of the opinion, based on experience with both types of assay, that, especially where a large number of assays are to be performed, paper chromatography, in the case of those amino acids for which it is readily applicable, may be generally as accurate and far more expeditious than are microbiological methods.

Acknowledgment

We acknowledge the interest and aid Professor Roger J. Williams has given in the preparation of this paper.

Summary

A paper chromatographic method for determination of amino acids in the presence of interfering substances such as are encountered in biological fluids has been described, and data relating to the accuracy and dependability of the method have been presented.

Analysis for certain amino acids in purified lactoglobulin has shown that, especially in the absence of interfering substances, analytical values accurate to ± 10–15% can be obtained by paper chromatography.

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Tissue Proteins and Carcinogenesis. III. Precancerous Changes in the Liver and Serum Proteins of Rats Fed Acetylaminofluorene [1]

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Introduction

Several well-defined changes in certain liver and serum proteins are associated with azo dye carcinogenesis in the rat. Miller and Miller (1) have demonstrated a firm binding of the azo dye to liver proteins which precedes the actual development of hepatomas. When the carcinogen, *p*-dimethylaminoazobenzene, was fed, Masayama and Yokoyama (2) and Price, Miller and Miller (3) noted that the concentration of desoxyribonucleic acid in the liver increased while that of ribonucleic acid decreased. In a recent study, we fed the active carcinogen, *m*′-methyl-*p*-dimethylaminoazobenzene, to rats and observed a progressive increase in the concentration of desoxyribonucleoprotein in the liver (4). The concentration of this component actually approached the high level found in hepatomas resulting from the azo dye. It was also observed that the liver content of riboflavin decreased while the liver globulin concentration increased significantly. From electrophoretic experiments, Cook, Griffin and Luck (5) found that the *m*′-methyl-*p*-dimethylaminoazobenzene resulted in an increase in the serum globulin and a decrease in the serum albumin.

The present study was initiated to determine whether structurally different carcinogenic compounds would induce the same changes in the liver and serum proteins as those induced by the carcinogenic azo dyes. Acetylaminofluorene has been reported to produce hepatomas and also tumors in other tissues (6,7). This compound was, therefore, fed to rats

[1] Presented in part at the fortieth annual meeting, The American Association for Cancer Research, Inc., April 17, 1949, Detroit, Michigan.

for a period sufficiently long to induce liver tumors. Analyses were made for desoxyribonucleoproteins, ribonucleoproteins, nitrogen, phosphorus, and riboflavin in normal rat liver, in precancerous livers, and in liver tumors resulting from the acetylaminofluorene. Also the serum proteins were determined by the electrophoretic method as this carcinogen was administered.

Methods

Male albino rats[2] of approximately 200 g. were fed diets containing 0.04% 2-acetylaminofluorene *ad libitum* for periods up to 24 weeks. At this time the remaining animals were maintained for 4 weeks on the diet free of the carcinogen. The purified basal diet was similar to that used by the Wisconsin group and was described in an earlier publication of this series (4).

At definite intervals, groups of from 4 to 6 rats were anesthetized with ether, and blood samples were taken by heart puncture. The livers were perfused *in situ* with cold 0.14 *M* NaCl. The excised livers were rapidly weighed and samples removed for moisture determinations. To the remaining liver was added 3.5 parts by weight of 0.4 *M* NaCl, and the mixture was homogenized for 2 min. in a Waring blendor. Total nitrogen concentration of the homogenate was determined by the micro method of Chibnall *et al.* (8); phosphorus, by the colorimetric method of King (9); and riboflavin, by fluorometric procedures (10,11). Desoxyribonucleoprotein (DNP) and ribonucleoprotein (RNP) were determined by the hot trichloroacetic acid method of Schneider (12). This same procedure was applied to liver tumors that resulted from the administration of the acetylaminofluorene.

The blood was allowed to clot, the serum pooled and diluted with 2 volumes of a sodium diethyl barbiturate buffer[3] of ionic strength 0.1, pH 8.3. The solution thus obtained was dialyzed with stirring for 15 or more hr. against 2 changes of the same buffer. The procedures were carried out at 1°C.

Analyses on the rat serum were carried out in a Tiselius apparatus at a temperature of 0.6°C. using an 11 cm. cell. The experiments were continued for approximately 5 hr. at potential gradients of 4–6 volts/cm. Initial and final boundary patterns were photographed by the Schlieren scanning method of Longsworth (13).

Results

Effect of Acetylaminofluorene on Certain Liver Components

Administration of the dietary acetylaminofluorene resulted in progressive damage of the liver. Mild cirrhosis was evident after the rats had been maintained 6 weeks on this regimen. At 20–24 weeks, the livers were enlarged and had a pronounced cirrhotic appearance. Small

[2] Holtzman, Sprague-Dawley strain.

[3] The sodium diethyl barbiturate buffer of Longsworth (17), ionic strength 0.1, and calculated to have a pH of 8.3: 0.050 *N* NaV; 0.020 *N* HV; 0.050 *N* NaCl, where V = diethyl barbiturate.

liver tumors were evident, however, after the animals had been on the experiment 24 weeks (Table I). Considerably larger tumors (probably cholangiomas) were evident at 28 weeks. During the last 4 weeks, the animals were fed the carcinogen-free basal diet. There was a slight initial increase in the DNP concentration of the liver as the diet containing the acetylaminofluorene was fed (Table I). After the initial response, however, the concentration of this fraction decreased to approximately 200 mg. (expressed as desoxyribonucleic acid) per 100 g. of liver at 20–24 weeks as compared to 273 mg./100 g. in the normal con-

TABLE I

Effect of 2-Acetylaminofluorene on Certain Liver Components

Purified basal diet + 0.04% 2-acetylaminofluorene[a]

Component	Control basal diet	Time rats were fed diet containing carcinogen						24-Week carcinogenic diet +4-week basal diet	
		2 wk.	4 wk.	6 wk.	14 wk.	20 wk.	24 wk.	Liver	Liver tumor
Av. liver weight, g.	9.0	9.1	10.0		11.0	19.0	20	18	
Liver appearance	Normal	Normal	Normal	Mild cirrhosis	Mild cirrhosis	Moderate cirrhosis	[b]	Cirrhosis liver tumors	
Nitrogen, mg.-%	2523	2590	2560	2380	2370	2410	2210	2150	1965
Phosphorus, mg.-%	291	339	207	301	270	292	290	287	228
Desoxyribonucleoprotein, as desoxyribonucleic acid, mg.-%	273	261	320	326	244	205	218	256	316
Ribonucleoprotein, as ribose, mg.-%	140	141	102	120	101	124	96	161	119
Riboflavin, γ-%	2540		2100		1670		1630	1430	320
Tissue moisture, *per cent*	75	73.5	73	74	75	73.4	70		

[a] The values are expressed on a fresh weight basis. Adult albino male rats. During experiment animals maintained weight or gained slightly.

[b] Livers enlarged, cirrhotic, small tumors evident.

trols. The RNP concentration also decreased from 140 mg. (expressed as ribose) per 100 g. of liver tissue to 100 mg. during this period. The nitrogen concentration of the liver decreased from 2523 to 2210 mg./100 g. liver. Both the DNP and RNP increased during the final 4 weeks of the investigation when the animals were maintained on the carcinogen-free diet.

From Table I, it may be observed that the phosphorus concentration and the tissue moisture remained relatively constant while the riboflavin concentration showed a progressive decrease as the acetyl-

aminofluorene was fed. The latter effect has been observed in other investigations with this carcinogen (14) and is of interest since the carcinogenic azo dyes also result in a lowered concentration of liver riboflavin. Liver tumors resulting from the acetylaminofluorene contained slightly more DNP than the precancerous liver (316 *vs.* 256 mg.-%) and a somewhat lower concentration of RNP, nitrogen, and phosphorus. Riboflavin concentration in the liver tumors was 320 γ-%, which agrees with the values reported for hepatic tumors by other investigators (15,16).

After the diets containing the acetylaminofluorene had been fed for periods from 20 to 24 weeks, the livers nearly doubled in weight as compared to the normal controls (Table I). Accompanying this increase in liver size was an overall decrease in the liver concentration of the DNP, RNP, nitrogen, and riboflavin. If expressed on a basis of total liver content, however, all of the components would show an increase. Specifically, the acetylaminofluorene resulted in liver hypertrophy and cirrhosis in which almost all liver components decreased somewhat in concentration but increased in total content because of the enlargement of the organ.

Serum Protein Studies

The electrophoretic diagrams were analyzed in the customary manner. Protein nitrogen for the 2 and 4 week serum samples were 7.44 and 8.50 mg. N/ml., respectively. The total refractive areas obtained from all of the groups indicated that the serum protein concentration did not change appreciably during the investigation. These values were also within the range found in several normal control groups. The electrophoretic values of the serum proteins were recorded as relative percentages in order to note changes during acetylaminofluorene carcinogenesis.

The data in Table II indicate that the concentration of albumin in the serum of rats on the acetylaminofluorene diet was within the range of values found previously (5) for normal sera except for the low 20-week and 28-week values. As the values for the percentage of serum albumin for rats fed the carcinogen for 6 weeks and for 24 weeks were normal, little significance can be attached to the low 20-week value. A low albumin value of 53% of the total serum protein was obtained for animals which had been on the acetylaminofluorene diet for 24 weeks followed by 4 weeks on a diet free of this compound. These animals had developed liver cirrhosis and liver tumors were evident at this time. Since a lowered relative amount of albumin is one of the changes

generally occurring in pathological sera, this low value for serum albumin at 28 weeks is probably significant.

The relative amount of α-globulin was found to be within the normal range throughout the entire period. However, the values did show an increasing trend as the feeding of the 2-acetylaminofluorene was continued; these values are above the average value found for normal sera.

TABLE II

Effect of 2-Acetylaminofluorene on the Percentage Composition of Rat Sera by Electrophoretic Analysis[a]

Time rats were fed diet containing carcinogen	Albumin	α-Globulin	β-Globulin	γ-Globulin
	per cent[b]	*per cent*	*per cent*	*per cent*
Basal diet[c]				
Range	60–74	11–26	10–14	2–10
Average of 6 samples	66	14	13	6
2 weeks: acetylaminofluorene	61[d]	16	14	9
6 weeks:	62	15	15	8
20 weeks:	53	15	21	11
24 weeks:	60	18	18	4
24 weeks: acetylaminofluorene plus 4 weeks: basal diet (tumors)	53	25	15	7

[a] Buffer[3]: sodium diethylbarbiturate; ionic strength: 0.1; pH: 8.3 ± 0.1; potential gradient: 4–6 volts/cm.

[b] Percentages are the fractional areas of the electrophoretic diagrams due to each component and represent the percentage of these components to the total amount of protein in the serum.

[c] Values for normal rat serum reported by Cook, Griffin and Luck (5).

[d] Percentages given at each interval on the acetylaminofluorene diet are for one pooled sample.

The percentage of β-globulin increased above the normal average value of 13% during the feeding of the acetylaminofluorene. The relative amount of γ-globulin remained well within the normal range during the period in which the carcinogen was fed and was also normal when tumors were evident.

During the later stages of the administration of the carcinogen, the blood serum was markedly turbid. Part of the observed increase of the α- and β-globulin components may have been caused by substances other than protein which migrated with the same velocity as these

fractions. It has been observed that lipides travel with the β-globulin in certain human pathological sera (17) and that they are often associated with the serum α- and β-globulins in various species (18). Visual inspection of the photographs obtained for the 20, 24, and 28-week samples, in which the turbidity was marked, showed that the turbidity boundary traveled with the α-globulin components. The turbidity component observed visually, at least, was not responsible for the increase in the percentage of β-globulin. The sum of the α- and β-globulin percentages showed an increase above the normal when the carcinogen was administered and displayed a definite upward trend as the feeding was continued (Table II). Feeding of the diet containing 2-acetylaminofluorene resulted in an increase in substances traveling with the velocities of β- and α-globulin, but no apparent increase in the relative amount of γ-globulin.

Discussion

Administration of acetylaminofluorene to rats resulted in a progressive damage and hypertrophy in the liver with a corresponding decrease in the concentration of DNP, RNP, nitrogen, phosphorus, and riboflavin. Liver tumors were evident at 24 and 28 weeks. These findings, compared to those obtained from a corresponding study carried out with the carcinogenic azo dye, *m'*-methyl-*p*-dimethylaminoazobenzene, (4) reveal that the two agents do not induce comparable precancerous liver changes. Comparative effects of the two carcinogenic agents are illustrated in Fig. 1. After the azo dye had been fed at a dietary level of 0.06% for a period of only 8 weeks, the livers were severely cirrhotic, the DNP had increased approximately 100%, the phosphorus had increased, the nitrogen and RNP had remained relatively constant, while the riboflavin concentration had decreased to 50% of the normal level. The azo dye brought about changes in certain of the liver components in a relatively short time and hepatomas were evident at 10 to 12 weeks. Administration of the acetylaminofluorene resulted in a gradual decrease in the concentration of liver components studied. No hepatic tumors were evident before 24 to 28 weeks. Hepatomas induced by the azo dye contained a relatively high concentration of DNP (over 700 mg.-%). The DNP concentration in the liver increased progressively as the azo dye was fed and actually approached the high level found in the resulting hepatomas (Fig. 1). In contrast to these findings, liver tumors resulting from the administration of

acetylaminofluorene contained only a slightly increased concentration of DNP (316 mg.-%) while the precancerous liver contained a lowered concentration of DNP (200 mg.-%).

The contrasting effects of the two carcinogens on liver DNP concentration conform to the dissimilarity of cells in the precancerous lesions produced by the two agents. Cox and associates (7) observed that liver nodules were the most common and prominent lesions which appeared during acetylaminofluorene feeding. The cytoplasm volume

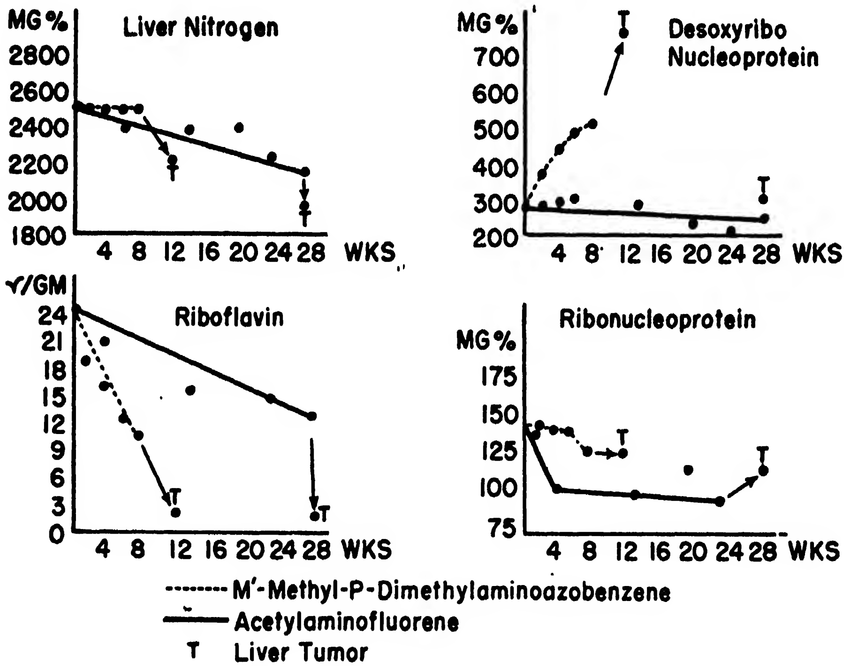

FIG. 1. Effect of two different carcinogens on certain liver components.

within these cells was distinctly greater than normal. On the other hand, hepatic parenchymal cells altered by feeding *m'*-methyl-*p*-dimethylaminoazobenzene have relatively large nuclei which appear to contain increased amounts of chromatin per cell. In addition, tumors produced by the two carcinogens are histologically dissimilar. Tumors induced by the azo dye present masses of closely packed cells with large, deeply-staining nuclei while liver tumors produced by acetylaminofluorene in this present study consisted largely of small duct-like structures lined by flattened cells with small nuclei.

Both the acetylaminofluorene and the *m*′-methyl-*p*-dimethylaminoazobenzene resulted in a decrease in liver riboflavin; however, the azo dye effect was more rapid. It is of interest to note that the carcinogenic activity of certain azo dyes is reduced by increased intakes of dietary riboflavin (19) while the activity of acetylaminofluorene is not influenced by this procedure (20). Both agents cause liver hyperplasia and damage. In the enlarged livers from rats fed the acetylaminofluorene, the DNP concentration was low. This component remained at a high level, however, in the livers from rats fed the diet containing the azo dye. This increase in DNP which is present in the liver cell nuclei appears to be of significance in azo dye carcinogenesis in view of the high concentration of DNP also observed in hepatomas resulting from this same agent. Data on the precancerous liver tissues and tumors resulting from the two different carcinogens would suggest that they do not induce tumors by the same mechanism or metabolic pathway.

It is of interest to compare the effects of acetylaminofluorene upon the composition of rat serum during the precancerous stages with the effects produced by certain of the azo dyes (5). The most active azo carcinogen studied, *m*′-methyl-*p*-dimethylaminoazobenzene, resulted in an increase in the γ-globulin fraction and a decrease in the albumin fraction of the serum at the end of 2 weeks of feeding. In contrast, prolonged administration of 2-acetylaminofluorene resulted in no increase in the relative amount of γ-globulin but led to an increase in the percentage of the α- and the β-globulin constituents. The albumin fraction showed a decrease as was also observed for the above mentioned azo carcinogen. These results indicate that the effect of acetylaminofluorene on the serum proteins differs from the effect produced by the azo dye.

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Summary

1. Livers of rats fed diets containing the carcinogenic agent, acetylaminofluorene, were analyzed at various intervals for desoxyribonucleoprotein, ribonucleoprotein, nitrogen, phosphorus, and riboflavin. Similar determinations were made on the liver tumors resulting from

this agent. Corresponding electrophoretic studies were made on the serum proteins.

2. The ingestion of acetylaminofluorene resulted in liver damage and hypertrophy. Liver tumors were evident at 24 weeks. During the precancerous period it was noted that the liver concentration of desoxyribonucleoprotein, ribonucleoprotein, nitrogen, and riboflavin decreased.

3. Feeding the carcinogenic acetylaminofluorene resulted in an increase in the percentage of the serum β-globulin. Other serum proteins remained relatively constant.

4. The effects of this carcinogen on the liver components and serum proteins are compared with the reported effect of the azo dye, *m'*-methyl-*p*-dimethylaminoazobenzene. The two agents both induce hepatic tumors but, from comparable studies of precancerous liver changes and serum protein observations, their modes of action must differ.

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The Effect of Cobalt on the Relationship Between Nucleic Acid Concentration and Growth Rate in *Proteus vulgaris* [1]

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Introduction

Cobalt, in relatively low concentrations, can prevent the growth of a large number of species of bacteria in a medium otherwise capable of supporting vigorous growth (12). The inhibition can be reversed by the addition of histidine, with which cobalt combines stoichiometrically (1). When cobalt is added to a growing bacterial culture, growth stops within one generation time. Concurrently, the heightened Q_{O_2} characteristic of the growing cells drops at least 50%. The respiration of resting cells, on the other hand, is unaffected by cobalt. Indeed, little or no effect of cobalt has been found on a variety of metabolic activities of resting cells of *Proteus vulgaris*. On the basis of these observations (13), it was thought that the action of cobalt might be a readily reversible interference with some process uniquely related to growth.

Cell reproduction and protein synthesis have been shown by Caspersson and others (2–6) to be related to nucleic acid metabolism. They have found, in a wide variety of cells, that wherever there is rapid synthesis of protein, there is a high concentration of pentose nucleic acid (PNA), and so postulated an important role for PNA in the synthesis of protein. Desoxypentose nucleic acid (DNA) has been accredited with playing an important part in the control of cell division. Malmgren and Hedén (6), using Caspersson's microscopic ultraviolet light absorption technique, have presented evidence for a correspondence of the concentration of total nucleic acid with the protien content of individual cells as well as with the rate of increase in cell

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number in bacterial cultures, including *P. vulgaris*. Using this organism, we studied the pattern of pentose nucleic acid and desoxypentose nucleic acid concentrations, by chemical means, as a function of the rate of growth of the bacterial culture. We also determined the effect of a growth inhibitory concentration of cobalt on these patterns.

Experimental

Materials and Methods

1. The organism used in this work was *Proteus vulgaris*.

2. *Nutrient broth* per liter: 3 g. meat extract (Difco), 10 g. Peptone (Difco), and 5 g. NaCl.

3. *Nutrient agar*: 3% agar in nutrient broth.

4. *Turbidity determinations*. Three milliliters of the inoculated broth whose turbidity was to be determined was added to 15 ml. of H_2O plus 0.2 ml. formalin. The turbidity was measured in a Pfaltz and Bauer fluorophotometer. The instrument was set at 100 with a Pfaltz and Bauer "vitamin B_1" glass standard. (If the turbidity of the culture was greater than 100 the B_1 standard was used to set the instrument at 50. The reading obtained with the sample was then multiplied by two in order to have all values comparable.) A corrective figure obtained by diluting 3 ml. of uninoculated broth with 15 ml. H_2O + 0.2 ml. formalin was subtracted from the experimental one.

5. *Nucleic acid Determinations*. For the determination of the nucleic acids, a slight modification of the method of Schneider was used (10). The bacteria to be analyzed were quantitatively removed from the suspending medium by centrifugation at 20,000 × g in a Sorvall SS-1 centrifuge for 10 min., washed twice with 40 ml. portions of H_2O, and made up to a volume of 25 ml. with H_2O. Two aliquots of 2 ml. each were removed for dry weight determinations. The remaining 21 ml.[2] was centrifuged and the residue extracted successively with 5 ml. of cold 10% trichloroacetic acid, 5 ml. of cold 10% trichloroacetic acid, 5 ml. of 80% alcohol, 5 ml. of 100% alcohol; then refluxed with 15 ml. of alcohol-ether (3:1), and dried *in vacuo*. The dried powder was washed with 2.5 ml. of 5% trichloroacetic acid, treated with 5 ml. of 5% trichloroacetic acid at 90°C. for 15 min. and again washed with 2.5 ml. of 5% trichloroacetic acid. The 10 ml. of combined trichloroacetic acid extracts was analyzed for pentose nucleic acid by von Euler's phloroglucinol reaction (11) and for desoxypentose nucleic acid by Dische's diphenylamine reaction (7). The trichloroacetic acid extract was also analyzed for total nucleic acid in the Beckman quartz spectrophotometer using an extinction coefficient of 22 for 0.1% solution of nucleic acid (4). The total nucleic acid concentration so determined was usually 80–90% of the concentration obtained by adding the two values obtained colorimetrically.

Results

Ten experiments were performed to determine the concentrations of pentose nucleic acid and of desoxypentose nucleic acid at various stages

[2] In one experiment 2 ml. was removed for nitrogen determinations.

of the growth cycle of *P. vulgaris*. These experiments differed only in minor details. A description of a representative experiment follows.

Cultures of *P. vulgaris* were grown at 37°C. in 2 Blake bottles containing nutrient agar. The bacteria were harvested after 17 hr. growth and suspended in 60 ml. of 0.05 *M* phosphate buffer, pH 7.3. Thirty-eight milliliters of the suspension was inoculated into 650 ml. of meat extract broth in a round bottom flask. The resultant turbidity was 40.9 (see *Methods*). The inoculated broth had approximately 10^9 viable cells/ml. The contents of the flask were aerated for 5 hr. at 37°C. Samples were taken for turbidity measurement after 0.5, 1, 2, 3, and 5 hr. At these times, 100 ml. of the culture was removed, treated with 2 ml. of formalin, and centrifuged at 20,000 × g for 10 min. The dry weight as well as the PNA and DNA content of the bacteria were determined. Results are given in Fig. 1.

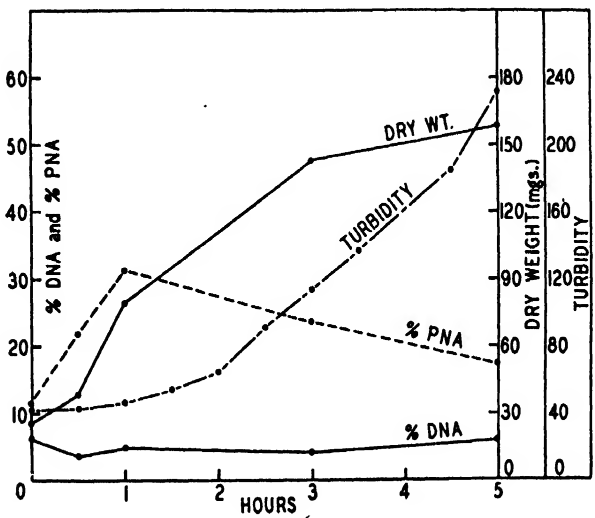

FIG. 1. Dry weight, turbidity, PNA concentration, and DNA concentration in *Proteus vulgaris* as a function of time in meat extract broth. The dry weight figures are expressed as mg./10 ml. of original inoculating suspension.

It will be seen that the dry weight as well as the turbidity of the culture increased rapidly. The percentage of DNA fluctuated. We do not think that the fluctuations were a result of experimental error in the determination of the amount of DNA since duplicate determinations of DNA content on aliquots of the same culture gave close agreement. It is of interest to note that Novikoff and Potter (9) noticed a similar fluctuation in DNA content in regenerating rat liver. The PNA concentration, on the other hand, began to increase almost immediately,

reached a maximum of roughly 2.5 times that of its initial value, and then declined.

A comparison of the synthetic activity of the bacteria, in terms of the percentage increase in dry weight/hr. with their concentration of PNA is illustrated in Fig. 2.

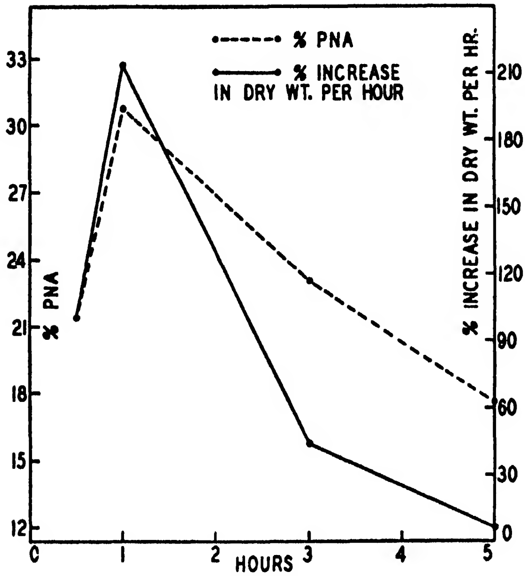

FIG. 2. Comparison of synthetic activity of *Proteus vulgaris* with PNA concentration, as a function of time in meat extract broth. The origin of the left hand ordinate has been shifted to allow for approximate juxtaposition of the two curves.

It can be seen that the two curves of Fig. 2 run roughly parallel to each other, especially during the early, most rapid stages of growth. When the rate of growth began to decline it did so more rapidly than did the concentration of PNA.

In the replicate experiments of the foregoing, similar results were obtained: the percentage of DNA fluctuated; the percentage of PNA always increased rapidly to 2–3 times the original value, showing a time course roughly parallel to the time course of the synthetic activity. In one experiment, the protein nitrogen content was determined. The rate of synthesis of protein was parallel to the rate of increase of dry weight. It should be pointed out that the concentration of PNA of the inocula for the 10 experiments varied from 6–12%. We have no satis-

factory explanation for this wide variation. However, no matter what the actual value of the PNA concentration in the inoculum was, a value of 2–3 times this original concentration was always attained when the culture showed maximum synthetic activity.

Effect of Cobalt

Cells of *P. vulgaris*, when inoculated into nutrient broth, swell in size, develop a heightened Q_{O_2}, and after an incubation period, begin to grow and divide (12,13). If cobalt is present in the broth at the time of inoculation, none of these changes take place.

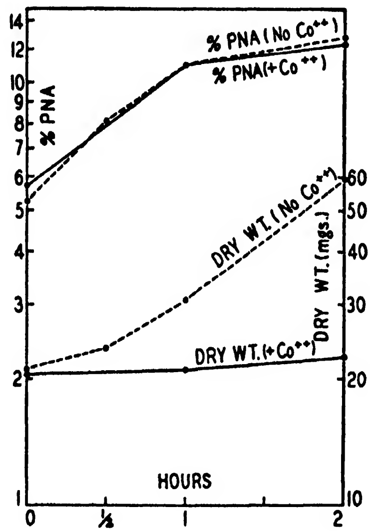

FIG. 3. Effect of cobalt on growth rate and PNA concentration of *Proteus vulgaris*, as a function of time in meat extract broth. Case I.

To determine whether the synthesis of nucleic acid by cobalt treated cells was also inhibited, we adopted an experimental procedure similar to that described in the previous section. In this case two cultures of *P. vulgaris* in meat extract broth were studied simultaneously. One flask contained cobaltous ions (added as the sulfate) at a concentration of 2×10^{-3} *M*. This concentration was sufficient to prevent growth. The other flask contained no cobalt and served as the control. The results of one of the experiments are given in Fig. 3.

In this experiment, it can be seen that even though the cobalt treated organisms did not grow, the course of the changes in PNA concentration was essentially identical with that of growing cells. This result was duplicated in two other experiments. The slight increase in dry weight in the cobalt treated culture can be accounted for by the increase in PNA concentration.

In three other experiments, using the same concentration of cobalt, the concentration of PNA did not increase to the same extent as it did in the control growing cells. Fig. 4 represents the minimal increase observed. It should be noted that in all cases, the cells treated with cobalt did not grow, although the PNA concentration did show a significant increase.

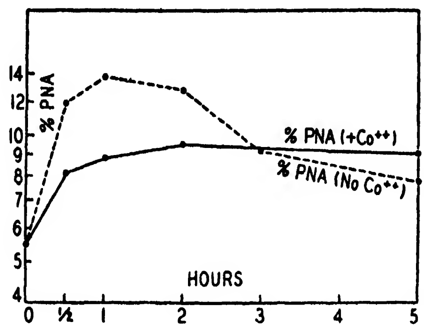

Fig. 4. Effect of cobalt on PNA concentration of *Proteus vulgaris*, as a function of time in meat extract broth. Case II.

The DNA concentration in the cobalt treated cells showed the same type of variation as did the DNA in the controls.

Discussion

The foregoing data show that rapid synthetic activity and growth are associated with a marked increase in PNA concentration in cells of *Proteus vulgaris*. Yet when growth and synthetic activity are prevented by cobalt, a similar increase in PNA concentration is still observed. Thus, while a high concentration of PNA may be necessary for synthetic activity that is represented by the increase in dry weight and protein, such synthetic activity is not necessary for increased PNA concentration.

If the increase in PNA concentration is a necessary step to the formation of protein, then the growth inhibitory effect of cobalt might be

attributable to an effect on the process by which PNA leads to protein synthesis.

Summary

The concentration of pentose nucleic acid in rapidly growing cells of *Proteus vulgaris* was found to be 2–3 times that in resting cells. The synthetic activity of the cells and the PNA concentration, as functions of time, were roughly parallel to each other.

When cobaltous ions were present in the nutrient broth, the cells did not grow. In spite of this, however, the pentose nucleic acid concentration increased markedly in such cells; in some cases to as great an extent as it did in the control growing cells.

The desoxypentose nucleic acid concentration fluctuated during growth, but on an average did not differ from the concentration found in resting cells. In the presence of cobalt, the desoxypentose nucleic acid concentration of the cells showed a fluctuation comparable to that of the growing cells.

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Effect of Cobalt on the Phosphorus Turnover Rate in the Nucleic Acids of *Proteus vulgaris* [1]

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Introduction

The pentose nucleic acid (PNA) concentration in growing cells of *Proteus vulgaris* increases rapidly during the early stages of growth (1). The synthetic activity of the organism (increase in dry weight or protein/hr.) and the PNA concentration, as functions of time of incubation in nutrient broth, are roughly parallel. When cobaltous ions were present in the nutrient medium no growth took place. The PNA concentration of such inhibited cells, however, increased to an extent comparable to that of the control growing cells.

If one assumes that PNA has an important function in protein synthesis, then the growth inhibitory activity of cobalt might be attributable to an effect on the metabolic process by which PNA leads to such synthesis. Since the turnover rate of the phosphorus of nucleic acids has been accepted as a measure of an aspect of nucleic acid metabolism[2] (6), we investigated the effect of cobalt on nucleic acid metabolism as reflected by such turnover rate.

Experimental

Methods

In using radioactive tracer techniques to determine the turnover rate of an element in a tissue component, which component remains con-

[1] This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[2] See, however, Brown *et al.* (13) for a suggestion that nucleic acid purine turnover may not necessarily parallel nucleic acid phosphorus turnover.

stant in amount during the experiment, the specific radioactivity of that component provides a measure of the turnover. To determine the turnover rate of any metabolic component in a culture of growing bacteria, however, it is necessary to consider that additional amounts of that component may be synthesized by the growing organism. If any significant amount of synthesis occurs, the specific radioactivity of the newly incorporated element will approximate the specific activity of that element in the nutrient material on which the organism is growing. The specific activity of the component isolated at the end of a given period of growth will be greatly influenced by the amount of the newly synthesized component and so can not be used directly as a measure of its turnover.

The turnover of any metabolic component in a culture of growing bacteria or in a culture in which the amount of that component changes during the course of the experiment can, however, be determined in the following way. Bacteria are grown on a medium containing the radioactive tracer and so contain labeled component molecules. An aliquot of a stock suspension of the resulting bacteria is inoculated into a growth medium containing no added radioactive material. After a suitable length of time, the bacteria are harvested and analyzed for the total amount of the element under question: in this case, pentose nucleic acid phosphorus and desoxypentose nucleic acid phosphorus. In addition, the specific radioactivities of these phosphorus fractions are determined. One therefore knows how much radioactivity, in the form being studied, is present in the bacteria at the end of the growth period. Similar analyses of the bacteria in another aliquot of the stock suspension give the amounts of such radioactivity initially present. The decrease in the total amount of radioacitvity in these fractions from the beginning to the end of the experiment is a measure of the amount of turnover.

Thus, the determination of the amount of phosphorus turnover in the nucleic acids of the cells of *P. vulgaris* inoculated into a growth medium, in the presence and absence of cobalt, was made from the following information:

1. The total weight of a powder prepared quantitatively from an aliquot of the bacterial stock suspension used for inoculation and from the bacteria of the culture after a suitable incubation period. This powder contained all of the nucleic acids of the bacteria.

2. The percentage of PNA and of DNA in the powder.
3. The percentage of phosphorus in the PNA and the DNA.
4. The specific radioactivity of this phosphorus.

From (1), (2), and (3) one can calculate the total amount of DNA phosphorus and PNA phosphorus in the bacteria, and together with (4), the total amount of radioactivity in these fractions.

The analytic methods used for the realization of the above four points follow:

1. *Preparation of the dried bacterial powder.* (All work up to the refluxing was done in the cold room at 2°C.) The bacterial culture to be analyzed was treated with 10 ml. of formalin/l. of culture, quantitatively centrifuged from the broth at 20,000 × g for 10 min. in a Sorvall SS-1 centrifuge, washed twice with 40 ml. of H_2O, and suspended in 50 ml. of H_2O. Two 2 ml. portions of this suspension were taken for the dry weight and 1 ml. for the total radioactivity of the bacteria. The remainder of the suspension was centrifuged at 20,000 × g for 10 min. and successively washed with 15 ml. of cold 10% trichloroacetic acid, 15 ml. of cold 10% trichloroacetic acid, 15 ml. of cold 80% alcohol, and 15 ml. of cold 100% alcohol. The residue from the alcohol wash was transferred quantitatively to a reflux flask with 15 ml. of alcohol and 5 ml. of ether, refluxed for 15 min., filtered, washed with ether, dried *in vacuo*, and weighed. The dry weight at this stage varied from 150–700 mg. This bacterial powder should be composed almost completely of proteins, phosphoproteins, and nucleic acids.

2. *The percentage of PNA and DNA in the dried bacterial powder.* 25.0 mg. of the powder was assayed for PNA and DNA by the method previously described (1).

3 and 4. *The percentage of phosphorus in the PNA and the DNA fractions, and the specific activity of this phosphorus.* For these purposes, it was necessary to separate the nucleic acids from each other and from other sources of phosphorus. This was done as follows: 200 mg.[3] of the bacterial powder, 400 mg. of finely ground Pyrex glass, and 3 ml. of 0.14 M NaCl in 0.05 M barbiturate buffer, pH 7.4 (henceforth called 0.14 NaCl-B), were ground for 1 hr. in a glass homogenizing tube (7) held in an ice bath. The suspension was transferred with an additional 3 ml. of 0.14 M NaCl-B to a 15 ml. Sorvall centrifuge tube and stirred overnight at 2°C. The following schematic diagram indicates the further treatment of the suspension.

The separated fractions were analyzed for PNA by von Euler's phloroglucinol reaction (8) and for DNA by Dische's diphenylamine reaction (9). Each nucleic acid fraction never had more than 5% of the other nucleic acid as a contaminant, and usually had less than 1%. The solutions gave negative biuret tests. It is unlikely that the hot 1 M NaCl would extract from the bacterial powder any phosphoproteins or any phospholipides that escaped extraction by the organic solvents. Further, the alkali treatment of the 1 M NaCl extract would hydrolyze any phospholipides or phosphoproteins present, leading to the production of inorganic phosphorus. Since

[3] As little as 100 mg. has been used. The exact amount is not important as this procedure is used solely for the preparation of some separated PNA and DNA, and not for quantitative determination of the total amounts present.

Procedure for Separation of Nucleic Acids from Proteus vulgaris

Bacterial powder. Ground and extracted with 6 ml. 0.14 *M* NaCl in 0.05 *M* Barbiturate (= 0.14 *M* NaCl-*B*)

Discard supernatant | Wash residue with 3 ml. 0.14 *M* NaCl-*B*

Discard supernatant | Residue

Add 4 ml. 1 *M* NaCl in 0.05 *M* Barbiturate (= 1 *M* NaCl-*B*)

Heat in boiling water bath 20 min., cool and spin, wash residue with 2 ml. of 1 *M* NaCl-*B*, spin, combine supernatants

Supernatant | Residue: discard

Add 0.6 ml. 6 *N* KOH and let stand overnight at 37° to hydrolyze PNA. Place in boiling H_2O bath for 5 min., cool and spin

Supernatant | Discard ppt.

Add 0.8 ml. 6 *N* HCl, get fine ppt., spin

Supernatant saved as PNA fraction[a] | To ppt. add 3 ml. H_2O and bring pH to 6–7. Add 6 ml. cold EtOH, get heavy white ppt., spin

Supernatant discarded | Ppt. dissolved in 3 ml. H_2O. Add 6 ml. cold EtOH, acidify with 6 *N* HCl, get a fine ppt., spin

Supernatant discarded | *Ppt. saved as DNA fraction*[a]

At suitable intervals during fractionation aliquots were removed for assay in the Beckman quartz spectrophotometer.

[a] Both PNA and DNA fractions brought to neutral pH and made up to 10 ml. volume with water.

there was no inorganic phosphorus present after hydrolysis, no phosphoprotein or phospholipides were extracted. The nitrogen content of both PNA and DNA was about 14–15%. A spectral curve of a typical separated fraction is given in Fig. 1. Usually, extinction values were determined only at 250, 255, 260, and 265 mμ. There was always a maximum between 255–265 mμ.

The percentage of phosphorus in each nucleic acid fraction was determined by the

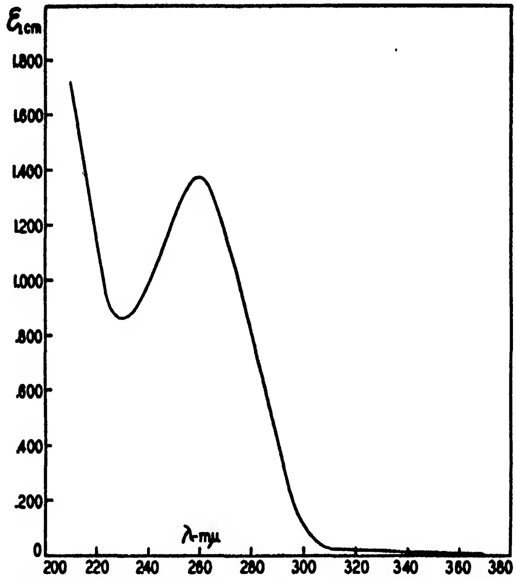

FIG. 1. Spectrophotometric curve of a separated nucleic acid fraction.

method of Fiske and SubbaRow (10). The phosphorus in the PNA was between 7–8%, while that in the DNA was between 5–7%. There was no inorganic phosphorus.

For the determination of radioactivity, an aliquot of the digested phosphorus sample plus 0.5 mg. carrier phosphorus as phosphate was precipitated as ammonium phosphomolybdate (11), the precipitate was filtered through a Tracerlab precipitation apparatus onto a Whatman No. 40 filter paper, mounted onto a Tracerlab ring and disc, and counted with a Tracerlab "64" scaler, using a 2.85 mg./cm.^{2} Geiger-Müller tube. A minimum total count of 3000 was obtained on all samples. Corrections for coincidence counting were applied.

Turbidity. The turbidity of the culture was determined as previously described (1).

RESULTS

Four similar experiments were performed. A detailed description of one will be given, and a summary of the other three will follow.

Proteus vulgaris was grown in 12 Blake bottles, each containing 75 ml. of nutrient agar[4] to which had been added 15 μc. P^{32}/l.[5] After 17 hr. of incubation at 37°C., the bacteria were washed off with 100 ml. of 0.9% NaCl, centrifuged at 20,000 × g for 10 min., washed twice with 100 ml. portions of 0.9% NaCl, and suspended in 100 ml.

[4] Per liter: 3 g. meat extract, 10 g. peptone, 5 g. tryptone, 30 g. agar, 5 g. NaCl; pH 7.6.

[5] The P^{32} was supplied by the Oak Ridge National Laboratory, Oak Ridge, Tennessee, through the facilities of the Atomic Energy Commission.

saline. Twenty-five milliliters of bacterial suspension was inoculated into 2 l. of meat extract broth[6] at 37°C. containing 200 p.p.m. of cobalt (3.2×10^{-3} M) as $CoSO_4$. Another 25 ml. was inoculated into 2 l. of meat extract broth at 37°C. without cobalt. This culture served as the control. The initial turbidities of the inoculated broths were 33. Each culture was divided into 2 one-liter portions. Each portion was transferred to a 2.8 l. Fernbach flask and incubated at 37°C. for 2 hr. The remainder of the inoculating suspension (50 ml.) was treated with 1 ml. of formalin, centrifuged, and the bacteria used for the determination of the amount of radioactivity in the PNA and DNA of the inoculum.

At intervals of 20 min., 3 ml. samples were removed from a control and a cobalt-treated culture, the bacteria centrifuged down, and the supernatant broth analyzed for total phosphorus and P^{32}. At the same time, samples were removed for turbidity determinations.

TABLE I

Calculation of Amount of Nucleic Acid Phosphorus Synthesized

	Weight of bacterial powder, mg.	% PNA[a] =	mg. PNA ×	% PNAP =	mg. PNAP	mg. new PNAP made	% DNA[a] =	mg. DNA ×	% DNAP =	mg. DNAP	mg. new DNAP made
Inoculum	170	15.0	25.5	7.9	2.01		5.2	8.84	6.0	0.530	
Cobalt	164	19.4	31.8	7.5	2.38	0.37	5.4	8.86	5.3	0.470	−0.060
Control	315	22.8	71.8	8.0	5.74	3.73	5.4	17.01	5.9	1.003	0.473

[a] These values are based on the weight of the acid-washed, defatted bacterial powder, not on the dry weight of the whole bacteria. It will be noted that the values for the PNA concentration of the control cells are not 2–3 times that of the inoculum, as reported in the preceding paper. However, the 2–3 fold increase in PNA concentration was found only in cells that were in rapid logarithmic growth; the cells in the current studies were not.

The turbidity of the control flask rose slowly and after 2 hr. reached 55. The turbidity of the cobalt-containing flask reached 38 after 0.5 hr. and remained at that figure for another 1.5 hr. After a total of 2 hr., the flasks were removed from the incubator, and 10 ml. of formalin was added to each flask. The total radioactivity of the PNA phosphorus and of the DNA phosphorus was then determined. Each nucleic acid fraction had less than 1% of the other nucleic acid as a contaminant. A summary of the resultant data is given in Tables I–III.

Table I shows the method of calculation of the amount of nucleic acid phosphorus in the bacteria and gives the results for the inoculum and for the cultures after incubation in nutrient broth, in the absence and presence of cobalt. It is clear, for example, from a consideration of the data given for PNA phosphorus that 3.73 mg. of new PNA phosphorus was produced during the period of incubation of the cells of the inoculum

[6] Per liter: 3 g. meat extract, 10 g. peptone, 5 g. glucose, 5 g. NaCl; pH 7.6.

in the broth minus cobalt while 0.37 mg. was produced by the cells in the broth plus cobalt.

To determine the amount of radioactivity derived from the radioactive phosphorus of the PNA originally introduced with the inoculum and still present at the end of the incubation time, the final specific activity (S.A.) of the PNA phosphorus must be multiplied by the total number of milligrams of PNA phosphorus recovered and then corrected for the radioactivity of new PNA phosphorus produced from the phosphorus of the growth medium.[7] Table II illustrates the procedure as applied to the PNA phosphorus of this experiment. Similar calculations were made for the DNA phosphorus of the bacteria in the inoculum and

TABLE II

Total Radioactivity of the PNA Phosphorus after Incubation, Corrected for Newly Incorporated PNA Phosphorus

	Control cells			Cobalt treated cells		
	PNAP[a]	c.p.m./mg. P	Total radioactivity	PNAP[a]	c.p.m./mg. P	Total radioactivity
	mg.	*(S.A.)*	*c.p.m.*	*mg.*	*(S.A.)*	*c.p.m.*
Recovered	5.74	16,788	96,363	2.38	32,027	76,124
Made	3.73	220	824	0.37	220	82
			95,539[b]			76,042[b]

[a] From Table I.

[b] Net radioactivity remaining from inoculated PNA-phosphorus.

after incubation in the presence and absence of cobalt. Table III summarizes the data obtained on the radioactivity of the nucleic acid phosphorus fractions for both PNA and DNA. The ratios of the amounts of radioactivity lost from the nucleic acids of the cobalt-treated bacteria to those lost by the controls are also given. It is evident that the amount of radioactivity lost from the PNA phosphorus in the cobalt-treated cells was 45% greater than that lost from the same fraction in the growing cells. The DNA phosphorus results are even more striking, the loss in the cobalt-treated cells being five times as much as in the control cells.

[7] The source of this radioactivity was the 0.9% NaCl in which the inoculating bacteria were suspended. The two saline washes that were given to the bacteria prior to inoculation did not completely remove the adherent P^{32}.

TABLE III

Loss of Radioactivity from Nucleic Acid Phosphorus

	PNA phosphorus[a]			DNA phosphorus[a]		
	Cobalt	Control	R^b	Cobalt	Control	R^b
Inoculated	139,346	139,346		36,027	36,027	
Recovered after correction	76,042	95,539		25,601	33,903	
Decrease	63,304	43,807	1.45	10,426	2,124	4.90

[a] Values shown represent total radioactivity in counts/min.
[b] Ratio of radioactivity lost from the nucleic acids of the cobalt treated cells to that lost from those of the control cells.

Three additional experiments were performed in a manner similar to that just described. A summary of the pertinent results obtained from these three experiments is given in Table IV. It can be seen that in all cases but one (Expt. 3, DNA phosphorus) the amount of radioactivity lost, and thus the amount of phosphorus turned over, is greater in the cobalt-treated cells than in the control cells.

TABLE IV

Loss of Radioactivity from Nucleic Acid Phosphorus
Total radioactivity in c.p.m.

Expt.	Inoculated	Recovered		Decrease		R^a
		Co	Control	Co	Control	
PNA phosphorus						
2	135,766	106,722	121,133	29,044	14,633	1.98
3	83,367	63,619	67,746	19,748	15,621	1.26
4	248,313	218,770	233,264	29,543	15,049	1.96
DNA Phosphorus						
2	53,952	12,160	48,759	41,792	5,193	8.05
3	30,949	30,609	29,192	340	1,757	0.194
4	109,151	58,020	86,723	51,131	22,428	2.28

[a] Ratio of radioactivity lost from the nucleic acids of the cobalt treated cells to that lost from those of the control cells.

Discussion

Because of the inherent error in each of the several analyses used in arriving at the final results, it is realized that these results cannot be regarded as having exact quantitative significance. Differences of less than 20% between the amount of radioactivity lost by the control and by the cobalt-treated cells may not be significant. In the results reported here, however, the loss of radioactivity from the PNA phosphorus of the cobalt-treated cells was nearly twice that of the controls, while the loss from the DNA phosphorus was about five times that of the controls. Hence, it is clear that cobalt had accelerated the loss of radioactive phosphorus from the PNA and DNA of the bacterial cells.

The decrease in radioactivity in the nucleic acid fractions of both the control cells and the cobalt-treated cells is not due to a loss of P^{32} from the cell during the incubation period. This is evident from two observations. The first is that the amount of radioactivity in the broth does not increase during the incubation time. The second is that the bacteria isolated at the end of the experiment still have the same total amount of radioactivity as they did when they were put into the broth.

Zilversmit *et al.* (12) outlined a method for the calculation of the turnover time of a compound from the rate of decrease of the specific activity of that compound. This method involves the assumptions that the turnover rate and the amount of compound present are constant throughout the experiment. For the estimation of the turnover time of nucleic acid phosphorus in bacteria in a growth medium the same method may be employed provided one assumes that the nucleic acid phosphorus introduced in the inoculum is analogous to Zilversmit's "constant amount of compound present." By considering that all the radioactivity in the phosphorus of the nucleic acids comes from this original amount of nucleic acid phosphorus, one can calculate the specific activity of this phosphorus at the beginning and at the end of the experiment.

Having made these assumptions and having treated our experimental results (Tables I and III) as required by Zilversmit's equation, namely,

$$\ln x/r = \ln C + T/T_t,$$

where

x = total radioactivity in c.p.m. at time T
r = mg. of nucleic acid phosphorus originally present
T_t = turnover time
C = specific activity at zero time

we found that the turnover time for the PNA phosphorus of the control was 12.2 hr. while that for the cobalt-treated cells was only 7.5 hr. The turnover time for the DNA phosphorus of the control cells was 32.9 hr. and for that of the cobalt-treated cells was 5.9 hr.

An increased formation of acid soluble mononucleotides at the expense of the formed nucleic acid is not considered a likely explanation of these findings, since the concentration of nucleic acid in the cobalt-treated cells increases significantly, in some cases as much as in the controls. It is probable therefore, that cobalt is directly or indirectly accelerating the transfer of nucleic acid phosphorus to its normal acceptor, or alternately, is diverting phosphorus to some abnormal acceptor.

Summary

1. Procedures and data evaluation necessary to the determination of phosphorus turnover rates in growing organisms are detailed.

2. A method has been developed for the separation from cells of *Proteus vulgaris* of pentose nucleic acid and desoxypentose nucleic acid in a moderately pure state.

3. The turnover rates of pentose nucleic acid phosphorus and of desoxypentose nucleic acid phosphorus of cells whose growth has been inhibited by cobalt are significantly greater than those of control growing cells.

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Enzymatic Reduction of Chloramphenicol (Chloromycetin[1])

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Introduction

During a recent series of experiments on the interrelationships of bacterial enzymes and the new antibiotic chloramphenicol, it was observed that bacterial cultures containing quantities of the antibiotic sufficient to prevent growth could when incubated for additional periods overcome the bacteriostatic effects of the drug so as to resume normal growth. These observations suggest that the bacterial cell must possess enzymatic systems which could convert the drug into some inactive form. With the establishment of the structure of chloramphenicol as D (−) threo-1-*p*-nitrophenyl-2-dichloroacetamide-1,3-propanediol (1, 2,3,4) and with the observations that the corresponding aminophenyl compound did not possess the same bacteriostatic properties as chloramphenicol, the possibility was suggested that bacteria might be able to inactivate this drug by enzymatic reduction of the nitro group to a primary aromatic amine group.

The present paper presents some of the observations which have been made as to the nature of this enzymatic reduction and the optimum conditions for the reaction.

Methods

The formation of aryl amine from chloramphenicol by enzymatic reduction was studied in both actively growing cultures and in suspensions of the washed cells of *E. coli, P. vulgaris, B. subtilis and B. mycoides*. All of the strains used were sensitive to the antibiotic (5).

The formation of aryl amine was followed by introducing 3 ml. of a chloramphenicol solution containing 2 mg. of the antibiotic per ml. into 5 ml. of an actively growing 18-hr. broth culture of the organism, or into a 2-ml. suspension of the washed bacterial

[1] Parke, Davis & Company's trade mark for chloramphenicol.

cells which were isolated from the broth cultures and suspended in 0.1 *M* phosphate buffer containing 0.85% sodium chloride. The samples were than incubated for 16 hr. at 37.5°C. and the reaction stopped by the addition of 5 ml. of 0.3 *N* trichloroacetic acid. The precipitated proteins were removed by filtration and the aryl amine present in the filtrate estimated by the Glazko *et al.* (6) modification of the Bratton *et al.* procedure (7) for the determination of aryl amine. The residue active chloramphenicol was determined by the microbiological method for the assay of chloramphenicol (5) and checked by the chemical method (6). In all cases the original chloramphenicol could be accounted for in terms of the aryl amine derivatives and the residual active chloramphenicol. The quantity of aryl amine present in the deproteinized solutions was determined by mixing a 1-ml. aliquot of the solution with 4 ml. of 0.25 *N* hydrochloric acid and 0.5 ml. of 0.1% sodium nitrite solution. After 5 min., 0.5 ml. of 0.5% ammonium sulfamate was introduced to destroy the excess nitrous acid present. Generally 3 min. was sufficient time for this reaction. The diazonium salt was then coupled to form a dye by the addition of 0.5 ml. of 0.1% solution of N-(1-naphthyl) ethylene diamine dihydrochloride. This reaction was essentially complete after 1 hr. at 37.5°C. and the quantity of dye formed could be estimated by reading the samples in a colorimeter at 525 mμ. If only traces of the aryl amine were present, they could be detected by using 4 ml. of the deproteinized solution and 1 ml. of 0.25 *N* hydrochloric acid.

The bacterial suspensions were prepared by growing the organisms in nutrient broth containing 3 g. beef extract, 10 g. peptone, 25 g. dextrose and 5 g. sodium chloride/l. of culture medium. The cultures were incubated for 18 hr. at 37.5°C. and the cells harvested by passing the culture through a Sharples centrifuge at 25,000 r.p.m. The cells were then washed three times with 0.8% neutral sodium chloride solution and suspended in 0.1 *M* phosphate buffer containing 0.85% sodium chloride. The suspensions used in these studies were prepared fresh for each determination and stored at 0°C. until used. Each suspension contained approximately 4 mg. of protein nitrogen per ml.

Experimental

In the initial experiments small concentrations (100–1000 μg.) of the antibiotic were introduced into 10 ml. cultures of *E. coli, B. mycoides, B. subtilis, and P. vulgaris.* The broth cultures were prepared by inoculating 9 ml. of the nutrient broth with 1 ml. of an 18-hr. culture of the organism. The cultures were then incubated for 6 hr. and the chloramphenicol solution introduced. The cultures were again incubated for 5 days at 37.5°C. and the quantity of aryl amine determined according to the procedure described above. Since all the organisms used in these studies were sensitive to the antibiotic, growth was immediately inhibited by the addition of the antibiotic. Therefore only those bacterial enzymes present in the cultures after the addition of the drug could be functioning in the reduction of chloramphenicol.

Results

The results obtained from the above experiments indicated that *E. coli* could convert approximately 40% of the chloramphenicol present to the corresponding aryl amine while *B. mycoides*, *B. subtilis*, and *P.*

vulgaris converted 8, 28, and 16% respectively. These rates of conversion were entirely too slow and too small a quantity of aryl amine was formed to permit an accurate estimation of the reactions involved.

By using heavy suspensions of washed bacterial cells and high concentrations of the drug, it was possible to study the enzymatic reaction of chloramphenicol using short incubation periods. The results obtained using this procedure appear to be identical with those results obtained using broth cultures. Some indications were obtained, however, that sub-bacteriostatic concentrations of the drug might be reduced faster in the actively growing cultures than was the case with cell suspensions. The exact velocity of this reaction could not be ascertained because of the extremely small concentrations of chloramphenicol that have to be used to obtain sub-bacteriostatic concentrations and the extremely small quantities of aryl amine produced under these conditions.

The optimum conditions for the enzymatic reduction of the nitro group of chloramphenicol by the bacterial suspensions tested were found to be pH 7.5, a temperature of 37.5–40.0°C. and a substrate concentration, *i.e.*, chloramphenicol, of approximately 2 mg./ml. of the test solution. The optimum pH range for this reaction appears to be rather limited. With pH values of 7 and 8, the reaction gave only 45 and 60% as much aryl amine, respectively, as when the reaction was carried out at pH 7.5. The formation of aryl amine increased fairly rapidly with an increase in temperature from 20–40°C. Additional increases in temperature to 50° and 60°C. produced a sudden drop in the conversion of chloramphenicol into the aryl amine derivative. This sudden drop in activity was probably due to the inactivation of the enzymes as a result of the long exposure (16-18 hr.) of the organisms to these higher temperatures. The influence of substrate concentration, chloramphenicol, was limited by the solubility of the drug in the test solutions. Only about 2 mg. of the drug could be dissolved in each milliliter of the solution.

The influence of various enzymatic substrates on this nitro reduction reaction was studied to determine, if possible, the nature of this reaction. There are at least two possible sources of the hydrogen used in the enzymatic reduction of the nitro group of chloramphenicol. It has been pointed out by Oppenheimer *et al.* (8) and Greville *et al.* (9) that it is possible to reduce certain nitro compounds by means of dehydro-

genases and their substrates. For example lactic acid and formic acid dehydrogenases can be used to reduce dinitrophenol. In these cases the hydrogen is transferred from the organic acids to the nitro compound by means of the dehydrogenase. The second possible source of the active hydrogen is from gaseous hydrogen. Certain bacteria possess the enzyme, hydrogenase, which can activate gaseous hydrogen which in turn can react with a hydrogen acceptor (10).

Of the various organisms known to be sensitive to chloramphenicol (5), probably more is known concerning the enzymatic systems of *E. coli* than of any of the other organisms tested. Therefore, this organism was selected for a more detailed study as to the reactions involved in the reduction of chloramphenicol by living organisms. There are four active dehydrogenases known to be present in *E. coli* cells: formic acid dehydrogenase, lactic acid dehydrogenase, succinic acid dehydrogensae, and glucose dehydrogenase (11,12,13,14); these can easily be determined quantitatively. The activity exhibited by each of these dehydrogenases present in *E. coli* cells will depend to a considerable extent on the conditions governing the growth of the organism and the procedure used in the preparation of cell suspensions. In the initial experiments it was found that growing the cells in cultures high in glucose (2.5%) and long centrifuging (1–2 hr.) at high speeds (25,000 r.p.m.) gave suspensions high in dehydrogenase activity. Moreover, the suspensions show considerable variation in the activity of each dehydrogenase tested.

The influence of each of these dehydrogenases on the enzymatic reduction of chloramphenicol was investigated by mixing 1 ml. of a 0.06 *M* solution of the dehydrogenase substrate with 2 ml. of the bacterial suspension and 3 ml. of a solution of chloramphenicol containing 2 mg. of the drug per ml. The samples were then incubated for 16 hr. and the quantity of aryl amine produced was estimated according to the procedure described above.

The various dehydrogenase activities of the suspensions were determined simultaneously by mixing 1 ml. of the substrate solution, 2 ml. of the bacterial suspension, 2.5 ml. of water, and 0.5 ml. of 1/5000 methylene blue solution in a Thunberg tube, under anaerobic conditions. The time for decolorization was then determined and used as an estimation of dehydrogenase activity (15).

Discussion

A typical set of data obtained from the above studies are shown in Table I. From this data there appears to be a relationship between the formation of aryl amine and dehydrogenase activity. The more active the dehydrogenase, as indicated by shorter time intervals for decolorization, the larger was the quantity of aryl amine formed. This would suggest that the hydrogen from the substrate was transferred to the chloramphenicol. In these cases, the chloramphenicol is probably acting as a hydrogen acceptor. It may be that the chloramphenicol is either the direct acceptor of the hydrogen or merely the last acceptor in a chain of oxidation-reduction reactions involving naturally occurring compounds present in the *E. coli* cell. Each substrate appears to have an optimum concentration level depending on the activity of the dehydrogenase being considered; see Fig. 1.

TABLE I

The Relationships between the Activities of Various Dehydrogenases Present in E. coli Cells and the Formation of an Aryl Amine Derivative from Chloramphenicol

Dehydrogenase	Dehydrogenase activity, in min.	Aryl amine formed from 100 μg. of chloramphenicol
Formic acid dehydrogenase	4.5	40.8
Lactic acid dehydrogenase	6.0	34.7
Glucose dehydrogenase	20.0	16.0
Succinic acid dehydrogenase	13.5	17.2
Control	—	15.2

It is also interesting to compare the stimulating effects of cysteine with the stimulating effects of lactate, formate, succinate, and glucose; see Fig. 1. Cysteine is known to have a stimulating effect on the hydrolysis of the amide linkage of chloramphenicol with the production of dichloroacetic acid and the free aliphatic base (16). The free base is furthermore reduced enzymatically to a corresponding aryl amine derivative. The free base appears to be reduced approximately twice as fast as chloramphenicol. For example equal molar concentrations of the free aliphatic base and chloramphenicol gave 222 and 118 μg. equivalents of aryl amine, respectively.

Cysteine appears to have a much greater stimulating effect than the other compounds tested. Apparently the velocity of the hydrolysis reaction is greater than the velocity of the reduction reaction. It is

interesting to note in this connection that the stimulating effects of cysteine and lactate, formate, succinate, or glucose are not additive when these compounds were used in combinations. The hydrolysis reaction appears to be the limiting reaction in the chain of reactions involving the hydrolysis of chloramphenicol and the reduction of the resulting free aliphatic base.

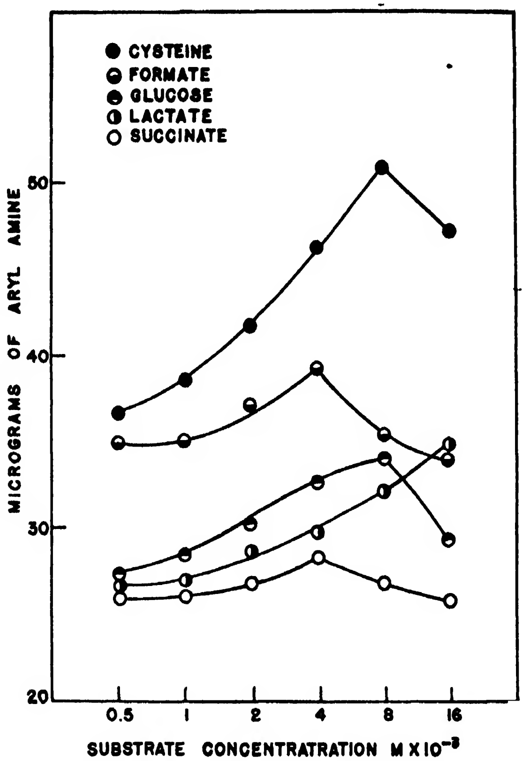

FIG. 1. The influence of various levels of cysteine, formate, lactate, succinate, and glucose on the reduction of chloramphenicol by washed *E. coli* cells.

In all the experiments involving cysteine, the possibility existed that the cysteine itself might be reducing the chloramphenicol under the conditions of the assay. This possibility was eliminated by running controlled experiments in which the living bacterial cells were replaced either with neutral sodium chloride solutions or with suspension of heat inactivated *E. coli* cells. The data from these experiments showed that neither the cysteine nor combinations of cysteine and lactate, for-

mate, succinate, or glucose could reduce the chloramphenicol in the absence of living bacterial cells.

The possibility that a hydrogenase might also be functioning in this reduction reaction was investigated by carrying out the reactions in an atmosphere of gaseous hydrogen. In this case, if a hydrogenase were involved, the gaseous hydrogen would be activated and could react with the nitro group present in the drug to form the corresponding aryl amine derivative. An atmosphere of gaseous hydrogen did not stimulate the enzymatic reduction of the antibiotic. This was true regardless of whether a pure atmosphere of hydrogen was used or 25% H_2–75% N_2, 50% H_2–50% N_2, 75% H_2–25% N_2 mixtures were used. Increasing the pressure of the hydrogen from one to two atmospheres also did not produce any significate increase in the quantity of aryl amine formed. Furthermore, gaseous hydrogen in combination with cysteine, lactate, formate, succinate, or glucose produced only those stimulating effects which could be accounted for in terms of the other organic compounds added to the reaction mixtures. From these observations it appears unlikely that a hydrogenase is functioning in the enzymatic reduction of chloramphenicol by washed *E. coli* cells.

Summary

The formation of an aryl amine from chloramphenicol by enzymatic reduction of the nitro group by bacterial cells has been studied to determine the optimum conditions for the reaction and if possible something of the factors involved in this reaction. *E. coli*, *B. mycoides* *B. subtilis*, and *P. vulgaris*, all of which are sensitive to this new antibiotic, are able to reduce the drug and thus destroy its bacteriostatic properties. Results obtained using suspensions of washed *E. coli* cells indicated that the conditions for the action which gave optimum reduction of the nitro group were a temperature of 37.5–40.0°C., a pH value of 7.5 and a concentration of approximately 2 mg. of chloramphenicol per ml. of solution.

The effects of various substrates such as formate, lactate, succinate, and glucose have been studied to determine if various dehydrogenases were involved in the reduction reaction. The results indicated that dehydrogenases are probably playing a role in the reduction of chloramphenicol by bacterial cells. Cysteine also stimulated the reduction reaction, but this effect can probably be related to the stimulating

effect of this amino acid on the enzyme involved in the hydrolysis of the amide linkage of chloramphenicol. The resulting free aliphatic base of chloramphenicol is reduced several times faster than the parent compound. Combinations of cysteine and the other organic compounds tested did not result in an additive effect of the individual stimulatory effects.

The data also show that a hydrogenase is not involved in this enzymatic reduction since gaseous hydroegn would not stimulate the reaction.

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The Relationship of Folic Acid, Vitamin B_{12} and Thymidine in the Nutrition of *Leuconostoc citrovorum* 8081 [1]

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Nutrition of Leuc. citrovorum

In a previous study it was observed that rats fed diets containing relatively high amounts of folic acid excreted in the urine a compound very active in stimulating the growth of *Leuconostoc citrovorum* 8081 (1). It was also noted that thymidine or high levels of folic acid permitted growth of the organism, but that the responses were different, both qualitatively and quantitatively, from that obtained with the highly active concentrates of the unknown growth factor (2,3). Shive *et al.* (4) observed that thymidine was more effective than folic acid in preventing the toxic effects of methyl folic acid on the growth of *Leuconostoc mesenteroides* 8293. Recently it was reported that thymidine could replace the vitamin B_{12} requirements of certain lactic bacteria (5–10). A detailed study was made on the relationship of folic acid, thymidine, and vitamin B_{12} in the nutrition of *Leuc. citrovorum* and on the differentiation of these nutrients from the citrovorum factor. Some of the results of this investigation are reported in the present paper.

Methods

The procedures and techniques employed in the studies on folic acid, thymidine, and vitamin B_{12} metabolism in *Leuc. citrovorum* were similar to those previously reported (1,2).

The growth of the organism was measured turbidimetrically with an Evelyn colorimeter after an incubation period of usually 18–20 hr. The folic acid contents of the liver and urine concentrates of the citrovorum factor (2) were determined microbiolo-

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gically with *Streptococcus faecalis* as the assay organism (1). The vitamin B_{12} contents of the concentrates were determined with *Lactobacillus leichmannii* (ATCC 4797) according to a procedure and medium similar to that employed by Skeggs *et al.* (9). The liver and urine concentrates were also analyzed for purines, pyrimidines, and thymidine by paper chromatography. The procedure employed was similar to that reported by Hotchkiss (11).

The liver concentrate used in this study was prepared from a commercial liver extract effective in the treatment of pernicious anemia. The procedure employed (3) consisted of repeated adsorption of the factor on charcoal at pH 3.0 and elution with dilute ammonia. The final concentrate (No. 1) contained approximately 11 citrovorum units/μg. of dry material. The "Citrovorum unit" (C.U.) was previously defined as the amount of substance required per ml. of culture medium to give one-half maximal acid production or growth of *Leuconostoc citrovorum* 8081 (2).

The rat urine concentrate (No. 2) was prepared in a manner similar to that employed in the preparation of the liver concentrates. Urine was collected from rats fed a complete synthetic diet containing 10 μg. of folic acid/g. Rats fed such a diet are known to excrete urine that is very active in stimulating the growth of *Leuc. citrovorum* (1). The final preparation contained 5–6 citrovorum units/μg. of dry material.

RESULTS

Toxicity of 4-Amino PGA for Leuconostoc citrovorum

In preliminary experiments it was observed that the omission of folic acid from the culture medium caused no apparent effect upon the response of *Leuc. citrovorum* to Reticulogen 360 (Parenteral Liver Extract with Vitamin B_1, Eli Lilly and Company), liver concentrates, rat urine concentrate, or thymidine. However, folic acid at a high concentration was previously found to replace the citrovorum factor (2,3). Analyses of Reticulogen indicated the presence of very small amounts of folic acid (8.7 μg./ml.) (2). Similarly, folic acid analyses of the liver concentrate employed in the present study indicated less than 4 μg. of folic acid/mg. of dry weight. Thus, it was apparent that the folic acid requirement of *Leuc. citrovorum* was very low or that the unknown factor replaced folic acid. In an attempt to answer this problem, studies were made involving the use of the folic acid antagonists, 4-aminopteroylglutamic acid (4-amino PGA) and methylfolic acid. Both 4-amino PGA and methylfolic acid were found to inhibit the growth of *Leuc. citrovorum* when cultured on a complete medium and in the presence of controlled amounts of Reticulogen. Methylfolic acid was, however, considerably less inhibitory than 4-amino PGA. 4-Amino PGA at a concentration of only 0.1 μg/ml. showed an appreciably toxic effect on *Leuc. citrovorum* when cultured in the presence of 40 μml. of

Reticulogen/ml. (Table I). At a concentration of 1.5 μg. of 4-amino PGA/ml., growth of the organism was completely inhibited. When the level of Reticulogen was increased sufficiently, the toxic effect of 4-amino PGA was overcome.

Although the culture medium contained 0.1 μg. of folic acid/ml., it was possible that the small amounts of folic acid present in the Reticulogen supplements was the effective agent in reversing the inhibition caused by 4-amino PGA. However, when the folic acid concentration of the culture medium was increased to levels as high as 6 μg/ml., little or no effect was obtained on reversing the inhibition (Table I). Thus, it was apparent that folic acid was not the active compound present in the Reticulogen.

TABLE I

Effect of 4-Amino PGA upon the Growth of Leuconostoc citrovorum 8081

4-Amino PGA	Reticulogen[a]	Folic acid	Galvanometer reading[b]
μg./tube	μml./tube	μg./tube	
0	0	1	97
0	200	1	77
0	400	1	50
0	600	1	36
0	1000	1	29
1	400	1	63
3	400	1	68
5	400	1	84
10	400	1	95
15	400	1	98
4	200	1	90
4	400	1	75
4	600	1	66
4	800	1	57
4	2000	1	29
4	400	20	73
4	400	40	73
4	400	60	72

[a] 1 C. U. (Citrovorum unit) = 25 μml. Reticulogen.

[b] Growth was measured turbidimetrically after an incubation period of 18 hr. (660 mμ. filter of the Evelyn colorimeter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay).

Similarly, the liver concentrate No. 1 was very effective in reversing the inhibition from 4-amino PGA (Tables II and III). One μg. of this concentrate counteracted the effects of 1–4 μg. of 4-amino PGA. The effectiveness of concentrate No. 1 was increased somewhat by increas-

TABLE II

Effect of Folic Acid and Concentrates of the Citrovorum Factor on 4-Amino PGA Toxicity for Leuconostoc citrovorum 8081

μg./tube	Galvanometer reading[a]				
	4-Amino PGA (μg./tube)				
	0	2	4	8	12
Liver concentrate No. 1					
0	100	—	—	—	—
1.16	67	78	89	100	100
2.32	41	56	64	89	100
3.48	35	47	57	80	99
5.80	—	36	47	68	91
11.60	—	29	38	48	72
Rat urine concentrate No. 2					
0	100	—	—	—	—
1.17	90	94	98	100	100
3.51	49	60	72	91	100
5.85	35	48	58	83	100
11.70	27	37	47	66	92
23.40	—	—	—	58	75
Folic acid					
1	100	—	—	—	
100	92	100	100	100	
200	85	99	100	100	
400	49	94	100	100	
800	35	67	87	97	

[a] Growth was measured turbidimetrically after an incubation period of 20 hr. (660 mμ. filter of the Evelyn colorimeter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay).

TABLE III

Effect of Thymidine and Liver Concentrates on 4-Amino PGA Toxicity for Leuconostoc citrovorum 8081

μg./tube	Galvanometer reading[a]			
	4-Amino PGA (μg./tube)			
	0	2	4	8
Thymidine				
0	98	—	—	—
2	59	61	63	67
4	46	47	47	56
6	40	42	41	48
10	36	41	36	45
Liver concentrate No. 1				
0	98	—	—	—
1.16	64	70	74	85
3.48	24	31	36	45
5.80	19	25	30	38
11.60	15	19	25	30

[a] Growth was measured turbidimetrically after an incubation period of 40 hr. (660 mμ. filter of the Evelyn colorimeter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay).

ing the incubation period from 20 hr. to 40 hr. (Tables II and III). The rat urine concentrate No. 2 was also very effective against 4-amino PGA. The effectiveness of the urine concentrate appeared to be related to its citrovorum activity. Based on citrovorum activity, the urine preparation was as active as the liver concentrate No. 1 against 4-amino PGA toxicity. This correlation indicates that the active principle in the liver and urine concentrates responsible for reversing the effects of the folic acid antagonists is the same as the compound required for the growth of *Leuc. citrovorum.*

High amounts of folic acid could replace the citrovorum factor and were also weakly effective against 4-amino PGA (Table II). Approximately 300 μg. of folic acid was required to counteract the effects of only 2 μg. of 4-amino PGA. Thus, the liver concentrate No. 1 was 100–

600 times more effective than folic acid against 4-amino PGA toxicity in *Leuc. citrovorum*. Thymidine was also effective in reversing the inhibition, but the response was variable and a longer incubation period was required to demonstrate the effect (Table III). Thymidine was less effective than the liver concentrate but much more so than folic acid.

Chromatographic Analyses of Concentrates

The possibility still remained that thymidine was the compound in the liver and rat urine concentrates responsible for stimulating the growth of *Leuc. citrovorum* and for counteracting the effects of 4-amino PGA. Experiments were undertaken with the use of paper chromatography to demonstrate the presence or absence of thymidine in these preparations. Samples of the concentrates and of known purines and pyrimidines were chromatographed on paper strips. The strips were then cut into segments and eluted with water, and the ultraviolet absorption at 260 mμ. measured with the Beckman spectrophotometer. Results of representative chromatograms are indicated in Fig. 1.

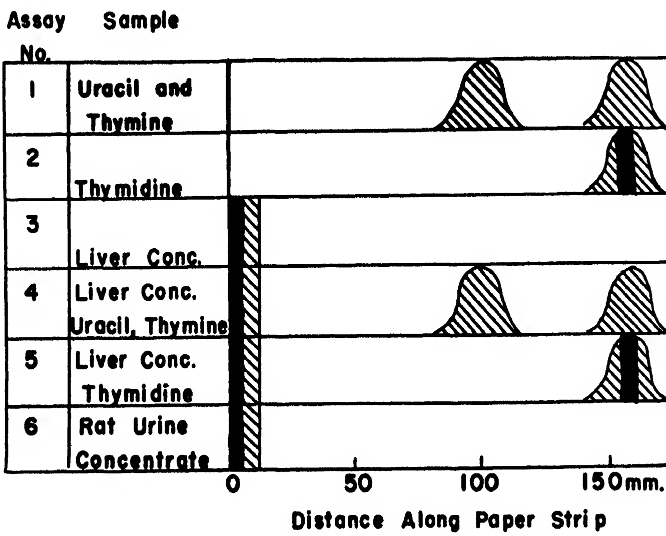

FIG. 1. Distribution of sample on paper chromatogram as determined (*a*) by ultraviolet absorption at 260 mμ. (shaded areas), (*b*) microbiologically with *Leuconostoc citrovorum* (solid bars). Assay: (1) 20 μg. uracil and 20 μg. thymine; (2) 10 μg. thymidine; (3) 23 μg. liver concentrate No. 1; (4) 23 μg. liver concentrate No 1., 20 μg. uracil, 20 μg. thymine; (5) 23 μg. liver concentrate No. 1 and 10 μg. thymidine; (6) 23 μg. rat urine concentrate No. 2.

Thymine, thymidine, and uracil were found to migrate approximately the same distances as reported by Hotchkiss (11). When the liver concentrate No. 1 or rat urine concentrate No. 2 was chromatographed, no bands, other than the initial starting band, were detected, indicating presence of little or no thymidine, purines, or pyrimidines. However, if known amounts of thymidine or of uracil and thymine were added to the liver concentrate, bands corresponding to the added known compounds were detected.

The eluates were saved and analyzed microbiologically for their ability to stimulate the growth of *Leuc. citrovorum*. The results are indicated in Fig. 1. When the chromatograms of the liver or rat urine concentrates were assayed microbiologically, the activity was present only in the eluates from the segments corresponding to the initial starting-point. When thymidine was added to the liver concentrate, a second area of activity was found corresponding to that when thymidine was chromatographed alone. From these results it appears that the liver and rat urine concentrates were free or very nearly free of thymidine and that some other compound was responsible for their activity.

Activity of Vitamin B_{12} and Other Related Compounds

Previously it was observed that vitamin B_{12} was ineffective in replacing the citrovorum factor (3). Crystalline vitamin B_{12} was ineffective in counteracting the toxicity of 4-amino PGA when tested at levels up to 0.1 μg./ml. The presence of enzyme hydrolyzed casein and Tween 80 in the medium caused no significant effect upon the response of vitamin B_{12}. The liver and rat urine concentrates appeared to be free of vitamin B_{12} when assayed microbiologically with *L. leichmannii*. Since this organism responds to thymidine, the absence of activity of the concentrates further indicates that thymidine was not present. Ascorbic acid, glutathione, choline, pyruvic acid, pteroyltriglutamic acid, or vitamin B_{12} in the presence of thymine were inactive in replacing the citrovorum factor. In contrast with the moderate activity of thymidine, the synthetic nucleosides, 1-D-glucosylthymine, 1-D-ribosylthymine, and 1-D-arabinosylthymine were ineffective in replacing the citrovorum factor. These compounds actually were slightly inhibitory towards *Leuc. citrovorum* when cultured in the presence of limiting amounts of thymidine.

Discussion

The ability of the concentrates of the citrovorum factor to counteract the toxicity of 4-amino PGA indicates a possible close relationship between folic acid and the factor. This becomes more evident when the ability of thymidine and high levels of folic acid to replace the factor are considered (2,3). Thymidine was active in reversing the toxicity of 4-amino PGA but the amounts required and the response obtained differed from that when the citrovorum concentrates were used. Furthermore, the concentrates were free of thymidine as determined by paper chromatographic and microbiological analyses. The results reported by Kitay *et al.* (12) on a strain of *Leuc. citrovorum* indicate that adenine desoxyriboside, hypoxanthine desoxyriboside, and cytosine desoxyriboside are also not involved.

Acknowledgments

The author is indebted to Dr. Karl Dittmer for samples of 1-D-glucosylthymine, 1-D-ribosylthymine, and 1-D-arabinosylthymine; to Lederle Laboratories for folic acid, 4-aminopteroylglutamic acid (Aminopterin), and a crude preparation of methylfolic acid (13); to Merck and Co. for crystalline vitamin B_{12}; to Atlas Powder Co. for Tween 80; to Dr. E. E. Snell, Dr. W. Shive and Dr. R. D. Hotchkiss for thymidine.

Summary

1. *Leuconostoc citrovorum* 8081 was found not to require folic acid when cultured in the presence of concentrates of the citrovorum factor or of relatively high concentrations of thymidine. However, in the presence of folic acid antagonists (4-aminopteroylglutamic acid or methylfolic acid) growth of the organism was inhibited. Concentrations of 4-amino PGA as low as 0.1 μg./ml. of culture medium produced a significant depression of growth.

2. The inhibition of growth by 4-amino PGA was readily reversed by the addition of concentrates of the citrovorum factor, prepared from liver extracts and rat urine. The concentrates were free of thymidine and low in folic acid.

3. High concentrations of folic acid were weakly effective against 4-amino PGA toxicity, while vitamin B_{12} was completely ineffective. Thymidine was active in reversing the toxicity, but the response and the amounts required were quite different from that when the liver concentrates were used.

4. One microgram of the liver concentrate was effective in reversing the inhibition of 1–4 μg. of 4-amino PGA, whereas 150 μg. of folic acid were required to reverse the effects of only 1 μg. of 4-amino PGA.

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Preformed Vitamin A in Crustacea

The source of the large quantities of vitamin A present in the liver of whales is still a matter of speculation. It is generally accepted that the crustacea ("Krill") forming their food contain little more than traces of active carotenoids (1,2). Zooplankton (3), copepods (2), and the shrimp *Penaeus foliaceus* (4) are said to have little vitamin A activity.

In the course of our work on the conversion of carotene to vitamin A in the intestine of the rat and of the pig (5,6,7) Professor B. C. P. Jansen of Amsterdam brought to our attention a publication by Wagner (8) not hitherto available in this country. In it Wagner, who worked at the Lopra whaling station in the Faroes, reported the isolation of 14.5 mg. crystalline β-carotene from 1 kg. "Krill" ("Gattung der Euphansia superba Dana") (*sic*). From a study of the concentration of vitamin A and carotene in the wall and contents of the alimentary tract of whales caught at Lopra, Wagner concluded that they obtained their vitamin A from carotene and that the conversion took place not in the liver but in the contents of the intestine.

Through the courtesy of Dr. R. Robinson of the whaling expedition of the *Balaena* to the antarctic we were able to obtain samples of stomach contents, consisting mainly of *Euphausia superba* Dana, and of various segments of the intestine of two fin whales. It should be pointed out that the crustacea examined by Wagner could not have been *Euphausia superba* Dana itself as this species is exclusively antarctic.

A portion of our "Krill" from the whale's stomach was extracted with a mixture of light petroleum and alcohol and the resulting deep red oil was saponified. Most of the color, due evidently to astaxanthin pigments, remained in the aqueous phase on extraction of the non-saponifiable residue with ether. The ethereal extract yielded only traces of β-carotene when chromatographed on alumina. On the other hand, the vitamin A alcohol fraction from the chromatogram (6,9) proved to contain appreciable quantities of a substance behaving typically like

vitamin A in the antimony trichloride reaction, showing an absorption maximum at 328 mμ. and biologically active in liver-storage tests with rats depleted of vitamin A. The concentration in the stomach contents was about 6 i.u./g. and that in the chromatographed non-saponifiable residue about 5000 i.u./g.

We have also examined a few samples of prawns (*Palaemon serratus*) bought locally and established that they also contain vitamin A itself to the extent of 1.0–1.7 i.u./g. As Grangaud and Massonet (4) had detected only very little vitamin A activity in the oil extracted from the flesh of the shrimp, *Penaeus foliaceus*, we analyzed separately the exoskeleton, the contents of the cephalothorax, and the tail of the prawns and found that the vitamin A-like substance, identified as for the "Krill," was concentrated almost exclusively in the exoskeleton which contained 3–5 i.u./g., or 2300–3800 i.u./g. chromatographed nonsaponifiable residue.

We hope to study shortly other crustacea including *Meganyctiphanes norvegica*, the "Krill" of whales of northern waters. Though our observations do not exclude the possibility that some of them contain enough carotene to supply the large stores of vitamin A in the liver of whales (in fact one of our samples of *Palaemon serratus* contained β-carotene in the exoskeleton to the extent of 0.5 μg./g.), and that this carotene is converted in the whale's intestine, they indicate another possible explanation of the still unsolved problem of the source of the vitamin A of whales.

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Influence of Certain Naphthoquinones on the Composition of the Lipides Formed by *Fusarium lini* Bolley[1]

Solanione, a pigment formed by *Fusarium solani* D_2 Purple (1), when added to the non-pigment producing mold, *Fusarium lini* Bolley, encroaches upon the growth of the organism, the total lipide content, the fat coefficient, and the composition of the isolated fat (2). Furthermore, some of these effects are not limited to solanione, but are also brought about by related naphthoquinones (3).

An attempt was now made to determine the influence, if any, on the characteristics of the fats obtained from *Fusarium lini* Bolley grown in the presence of certain naphthoquinones; *e.g.*, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone (menadione), and 2-hydroxy-3-methyl-1,4-naphthoquinone (phthiocol). One mg. of each of the quinones was added to 1 l. of a Raulin-Thom medium containing 2½% glucose, which was subsequently inoculated with a suspension of a 3-day old culture of *Fusarium lini* Bolley (4).

After a growth period of 21 days, the mats were dried and extracted with petroleum ether. The iodine values of the petroleum ether-isolated fat were determined by the Hanus method (5). These values, together with other pertinent data are recorded in Table I.

TABLE I

Effect of Certain Naphthoquinones on Fat Formation in Fusarium lini *Bolley*

Pigment in media	Mycelial weights	Fat coefficients	% Total lipide	Iodine values	% Sterol in isolated fats
1 mg./l.	*g.*				
Naphthoquinone	3.35	0.83	6.20	163	12.3
Menadione	2.26	0.53	5.87	142	8.7
Phthiocol	1.79	0.53	7.45	153	9.2
Control (no pigment)	4.20	1.23	7.31	152	14.3

Since the changes of the iodine values show an opposite trend, as compared to the control, it was thought advisable to determine the amount of ergosterol present in the isolated fats. This was done according to a method previously employed (6). These data are also recorded in Table I.

[1] This study is being carried on under the auspices of the Research Corporation and the Office of Naval Research.

It is known (7) that ergosterol can give an iodine value as high as 400 by Dam's method. Therefore, the iodine value of ergosterol was determined by means of the Hanus method. The value obtained was 436. It is apparent that this high value was not without influence on the iodine values of the isolated fats. Therefore, the iodine values of the fats were determined after removal of the ergosterol by saponification.

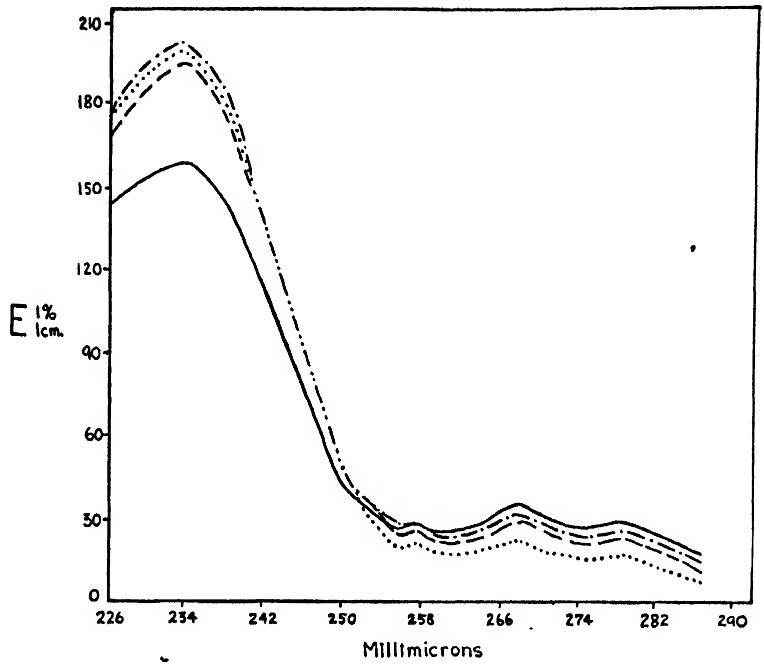

FIG. 1. Ultraviolet absorption curves of isomerized fats extracted from mycelia grown in the presence of: - - - - -naphthoquinone; · — · — · — phthiocol; ······· menadione; ———— control (no pigment).

The method of saponification was controlled so as to prevent any isomerization of the unsaturated fats (8). The values of the saponified fats are summarized in Table II.

TABLE II

Iodine Values of Saponified Fats

Pigment in media 1 *mg./l.*	Iodine values
Naphthoquinone	99
Menadione	98
Phthiocol	103
Control (no pigment)	86

It can be seen from these data that the effect of the addition of the pigments is to increase the desaturation (9) of the fats formed and to affect the amount of sterol formed.

Furthermore, from the accompanying spectrophotometric curves of the isomerized, sterol-free fats it can be recognized that linoleic acid (absorption maximum at 234 mμ) and linolenic acid (absorption maximum at 268 mμ) are not the only unsaturated acids responsible for the differences in the iodine values. It would appear that the percentages of oleic acid present in the mats grown in the media to which pigment has been added are higher than that of the control.

These results indicate the possibility of arbitrarily influencing the composition of biologically formed fats, and the existence of a relation between the effectors and the dehydrogenations occurring in the course of the carbohydrate → fat conversion by molds.

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Book Reviews

Chemistry of Specific, Selective, and Sensitive Reactions. By Fritz Feigl, Laboratory of Mineral Products, Ministry of Agriculture, Rio de Janeiro, Brazil. Formerly Professor of Analytical and Inorganic Chemistry at the University of Vienna. Translated by Ralph E. Oesper, Professor of Chemistry, University of Cincinnati, Ohio. Academic Press Inc., New York, N. Y., 1949. xiv + 740 pp. Price $13.50.

During recent years the problems of analytical chemistry have become increasingly complex. This is due largely to the fact that the methods of analysis have been extended to an ever larger number of materials, both of natural and synthetic origin, and also to the fact that there is a continuing effort to detect and determine minimum quantities of different substances in these materials. In short, according to Dr. Feigl, "the goal of analytical work must be the identification and determination of the smallest possible quantity of a substance, even in the presence of other substances, using a minimum of material, time and working efforts, regardless of the technique involved." In view of this objective, the specificity, selectivity, and sensitivity of an analytical process is of the greatest possible importance. In the past much of our analytical research has been largely of an empirical nature, with a minimum of systematic design, and this has been due largely to a lack of understanding of the fundamental processes and of the nature of the materials which are involved in analytical procedures. This has been a serious handicap to the progress of analytical chemistry.

In Dr. Feigl's book we have one of the most important modern contributions to the literature of analytical chemistry. According to the author, this book is "an attempt to summarize our knowledge of the scientific background of the specificity, selectivity, and sensitivity of analytical procedures." The book is not simply a bibliography of chemical methods for the detection and determination of various substances, but rather presents a discussion of such regularities and rules as govern specificity, selectivity, and sensitivity of analytical procedures. To this end the mechanism of chemical reactions, the composition and constitution of reacting substances and products obtained, and the conditions under which the reactions take place are presented. In addition, the relationship between solubility, color, fluorescence and other properties are considered. Thus the chemist is provided with a guide for the systematic search for new methods of analysis and for the improvement of existing ones.

The material of this book is presented in a clear, vigorous style and with all subjects well illustrated with numerous examples selected from analytical procedures and from the chemical literature. Approximately 1100 references to the literature are included for the benefit of those who desire additional information.

The book is divided into 12 chapters. Chapter I (6 pages) consists of general comments on the analytical usefulness of chemical reactions. Chapter II (16 pages) discusses the characterization of chemical tests by sensitivity, selectivity, specificity,

and limiting proportion, and the role of reaction conditions. Chapter III (44 pages) describes the types of complex and coordination compounds which play a role as reactants and products of reactions in analytical processes. Chapter IV (42 pages) is concerned with masking and demasking of reactions. Chapter V (58 pages) contains a discussion of methods for increasing the reactivity of systems by complex-formation, catalyzed and induced reactions, induced precipitation and induced solution. Chapter VI, which is very long (202 pages), contains an excellent discussion of the application of organic reagents in inorganic analysis. Particularly, the effects of certain atomic groupings on the specific and selective action of compounds are described. Since the very numerous organic compounds provide the most promising source of new analytical reagents, and since because of the very great number of these compounds the search for new reagents has necessarily been slow and laborious, this chapter will be especially useful to the research analyst. Chapter VII (14 pages) is similar in plan to the preceding chapter except that the effect of certain atomic groupings on the specific and selective activity of compounds in organic analysis is considered. Chapter VIII (28 pages) covers the regularities and anomalies in the solution of materials in indifferent solvents. Chapter IX (22 pages) contains an excellent discussion of the influence of size- and weighting-effects on solubility and salt-forming ability. Chapter X, also very long (180 pages), covers with great thoroughness the role of surface effects in analytical chemistry. This includes a study of color, solubility, and suspendibility of solids as a function of particle size; protective colloid and peptization action; colloid masking and sensitization by organic compounds; adsorption as a general phenomenon of the accumulation of materials on surfaces; modification of the reactivity of materials by adsorption; adsorption of dyes and other compounds; and the isolation and separation of materials by adsorption (capillary analysis, chromatography, and foaming analysis). Chapter XI (58 pages) consists of a discussion of the genetic formation of materials and topochemical reactions. Chapter XII (24 pages) describes the analytical use of fluorescence effects and photochemical reactions. An author index and an excellent subject index are also included.

The book is attractively bound in green cloth, is on good quality paper, and the arrangement and typography are excellent. A very liberal use of structural formulas contributes materially to the effectiveness with which the subject matter of this book is presented.

All chemists are greatly indebted to Dr. Feigl for the immense amount of material collected in preparing this book, and for the critical evaluation and interpretation of these data. Dr. Oesper is also to be complimented on his fine translation of the German manuscript. This book is recommended as indispensable to all students and chemists engaged in research in analytical chemistry, and to all those who have need of the knowledge of fundamental principles involved in analytical procedures.

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Isotopic Carbon. By M. CALVIN, C. HEIDELBERGER, J. C. REID, B. M. TOLBERT and P. F. YANKWICH. Wiley & Sons, N. Y., 1949. xiii + 376 pp. Price $5.50.

Five staff members of the Radiation Laboratory at the University of California have collaborated in writing a book intended primarily as a manual for all groups using, or contemplating the use of, isotopic carbon. The considerable experience of

the authors guarantees that much valuable information is collated on assay techniques and manipulation of the carbon isotopes. There are 12 chapters with 9 special appendices and a bibliography of tracer carbon applications complete to July, 1948.

After an introductory chapter dealing with production and properties of isotopic carbon, there is a short chapter of 5 pages describing in a general way the measurement of the stable isotope, C^{13}. Other material on techniques involving this isotope is included in other parts of the book but a really adequate discussion of this topic is not given. This is regrettable because information about C^{13} assay is much less widespread than that on the assay of the radioactive isotopes to which the great bulk of the book is devoted. Chapter 3 presents a discussion of the radiation properties of the carbon isotopes. The material on self-absorption is particularly detailed and should be very helpful to beginners. Chapters 4 and 5 are reserved for a discussion of instrumentation. It is not clear why this material was divided into two separate chapters entitled "Instruments for Radioactivity Measurements" and "Detectors for Radioactivity Measurements." One would suppose there is little distinction of this kind to be made. These chapters contain a large amount of material on design, construction and manipulation of assay equipment. Much of this is gathered together for the first time and some has been available previously only by word of mouth. The same remarks apply to Chapters 6 and 7 on sample preparation.

The eighth chapter, on vacuum techniques, is perhaps the weakest section. A rather large number of loosely-worded statements, as well as some misstatements, occur. From the standpoint of the novice in vacuum technique this chapter is decidedly inadequate. Perhaps the most unfortunate aspect is the very definite impression, not intended but given, that without a multiplicity of complicated glassware and electronic gadgets work with C^{14} is difficult. The writer of this chapter tried specifically to avoid this notion with a statement on p. 128 that a "vacuum system need be neither elaborate nor expensive," but the ensuing 20 pages do little to allay the fears of the beginner.

The strongest section of the book is made up of the next four chapters. In Chapter 9, a collection of all syntheses involving C^{14} up to April, 1948, as well as some unpublished procedures, is presented. The detail is adequate and the clarity much improved over some of the previous chapters. Chapter 10 discusses criteria of purity and is commendable in pointing out some of the hazards encountered in work with isotopes. It would have been more useful, however, if some specific data and less generalized discussion had been offered. Chapter 11 deals with degradation procedures, and is of the same good quality as Chapter 9. One omission from the list of degradation procedures may be noted, namely the Barbier-Wieland procedure used by E. Stadtman and H. A. Barker at the University of California for degrading caproic acid. Chapter 12 is a useful summary of biosynthetic procedures.

The appendices present short but detailed sections on isotope dilution methods, statistics of counting data, coincidence corrections, counting efficiency, self-absorption data, and vacuum apparatus.

This reviewer circulated the book among a number of his colleagues representing various degrees of sophistication with respect to work with tracer carbon. The comments which were received quite definitely indicated that this book would be a valuable addition to tracer literature, but that the ministrations of a critical copy

editor were needed to tighten the style so that rather frequent occurrence of ambiguities and confused statements might be eliminated.

MARTIN D. KAMEN, Saint Louis, Mo.

Biochemical Society Symposia No. 2; The Biochemical Reactions of Chemical Warfare Agents. Edited by R. T. WILLIAMS. Cambridge University Press, Cambridge, England, 1948. iv + 73 pp. Price 5 s.

In this monograph, one finds concisely summarized the brilliant contributions made by the British biochemists during the last war to our understanding of the biochemical action of the so-called war gases. Much of the work described herein has already found its way into public print, but some of it appears for the first time stripped of the bold letters, "TOP SECRET," that crowded every page of the confidential documents that circulated among some of us during those "darkest hours." This is not a monograph to be put aside until another war perhaps makes its contents imperative reading. There is much of value to be found in it for the pharmacologist and biochemist, especially if he is interested in the interaction of enzymes and drugs.

This is the first time that the reviewer has seen available to the general public a description of the outstanding contribution made by A. G. Ogston to the kinetics of replacement reactions in aqueous solutions of mustard gas. The importance of this contribution cannot be overemphasized, since, with his development of the term competition factor (*F*), it became possible to evaluate for the first time the extent of reactions of mustard gas in aqueous solution with any known compound. As a result, extensive tables were compiled, in both England and the U. S. A., which permitted a comparison of the relative affinities of mustard gas for a large series of both naturally occurring and synthetic compounds. The affinity of some compounds for mustard gas is so great that, at the proper concentration, they can monopolize its reaction and so protect less active compounds from attack. These kinetic findings offered high hopes of achieving therapeutic effects which, however, did not materialize for reasons which cannot be developed here. Those with a current interest in the mustard gases from the standpoint of production of mutants, the treatment of leukemia, *etc.*, might do well to delve a little more deeply into this important aspect of the action of the mustard gases.

The chapter by Ogston is followed by two on the reaction of mustard gas with proteins (by J. C. Boursnell) and enzymes (by D. M. Needham). The work reviewed in these two chapters shows that many groups in the protein molecule are capable of undergoing replacement reactions with mustard. The relative affinities of these groups for mustard is, however, not yet clear. Moreover, the activity of certain enzymes is unaffected by treatment with mustard while others are easily inactivated. The reason for the difference between a mustard-sensitive or insensitive enzyme active group has not been elucidated. Another valuable tool is thus at hand to pry into the configuration of protein molecules.

The chapter on reactions of arsenicals by R. H. S. Thompson, and one by M. Dixon on reactions of lachrymators, summarize the no small contribution made to our knowledge of —SH groups in proteins by the study of these chemical agents of warfare. The work of the Oxford biochemists under the leadership of R. A. Peters, which resulted in the discovery of British Anti-Lewisite (B.A.L.), is discussed by Thompson

and the chemical value of this compound in heavy metal poisoning is pointed out. The work of the Cambridge school on lachrymators is summarized in the words of Dixon as follows:

"All these different lines of evidence show that, at any rate, lachrymators of type (a) (halogen-containing) in neutral aqueous solution are highly specific reagents for —SH groups in proteins. In consequence, they have given us what is in my opinion the most reliable test for identifying —SH enzymes, and also a promising method for the estimation of —SH groups in proteins."

The reaction of fluorophosphonates with esterases is dealt with by E. C. Webb. The parasympathomimetic and toxic action of these compounds continues to elicit great interest among nerve physiologists. Little is yet known, however, of the mode of reaction of these compounds with the enzymes they attack nor of the type of group in the enzyme molecule which is so susceptible to their action.

The final chapter deals with the pharmacology of the chloroethylamines by E. Boyland, in which the similarity of their biological properties to ionizing radiations is most strongly stressed. This contributor strikes the keynote of this monograph when he says:

"One might have expected that the substances developed for use as chemical warfare agents would have been merely the starting-points for compounds with particular peacetime uses. In effect, however, the chemical warfare agents are themselves finding application. In this field, the 'swords of war' have required almost no chemical research work before their use as 'plough shares.'"

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Chemistry of the Carbohydrates. By WILLIAM WARD PIGMAN, The Institute of Paper Chemistry, Appleton, Wisconsin, and RUDOLPH MAXIMILIAN GOEPP, JR., The Atlas Powder Co., Wilmington, Delaware. Academic Press Inc., New York, 1948. xvii + 748 pp. Price $10.80.

This book is a monograph on the broad field of carbohydrate chemistry "from the organic, physical, analytical, biological, and industrial chemical aspects."

Chapter I (22 pp.) gives a brief survey of the historical development of carbohydrate chemistry, its general chemistry from the standpoint of stereoisomerism, activation by carbonyl groups, intermolecular reactions, the glycosidic hydroxyl, and polymeric carbohydrates, together with nomenclature and definitions. Chapter II (66 pp.) deals with the structure and stereochemistry of the monosaccharides, including the ring structure of the sugars, their tautomerism in solution, and the optical superposition rules. Chapter III (60 pp.) treats of the occurrence, properties, synthesis, and analysis of the individual monosaccharides. It begins with the naturally occurring monosaccharides (hexoses, pentoses, methyloses, heptoses) followed by the syntheti monosaccharides and is concluded by the qualititave and quantitative methods determination. In Chapter IV (37 pp.) the esters of monosaccharides with orga acids, with inorganic acids and with both together (halogenoacetyl sugars) are de cribed. In Chapter V (45 pp.) the glycosides are treated. These include the glycosa (inner glycosides), full acetals and thioacetals and the condensation products of simp carbohydrates with aldehydes and ketones (acetone sugars). In Chapter VI (46 pp presents the chemistry of the polyols, both the acyclic polyols as well as the inosito

and related compounds. In Chapter VII (57 pp.) the acids and other oxidation products of carbohydrates are described. In this chapter aldonic acids, saccharic acids, uronic acids, keto-aldonic acids, ascorbic acids and osones are found. Chapter VIII (30 pp.) deals with the ethers, anhydrides (inner ethers) and unsaturated derivatives of the monosaccharides. In Chapter IX (52 pp.) the nitrogenous derivatives of the simple sugars are summarized. N-Glycosides—nucleotides, nucleic acids—hydrazones, oxazones, oximes, amino sugars (glucosamines), and compounds of sugars and amino acids or proteins are described. With this chapter the chemistry of the simple sugars, the monosaccharides and related compounds concludes.

In Chapter X (32 pp.) the chemistry of oligosaccharides, up to the tetrasaccharides, follows. Chapter XI (53 pp.) starts with a description of the naturally occurring glycosides (the important ones) including streptomycin, and then considers the more important glycosidases, especially those of almonds and yeast, together with the kinetics and theories of their action.

Chapter XII (17 pp.) begins with the chemistry of the polysaccharides by presenting the general basis of their classification and structure. Chapter XIII (31 pp.) gives a view of cellulose chemistry and its more important derivatives. Chapter XIV (42 pp.) brings the starches and starch substances (glycogen) with a paragraph on the action of enzymes on starch. Chapter XV (47 pp.), the last, deals with the polyuronides, hemicelluloses, plant gums, microbial polysaccharides and related substances, including a paragraph about glycoproteins.

The book contains an Author Index (20 pp.) and a very carefully and completely written Subject Index (80 pp.).

A large number of tables and figures elucidate the text.

Since the inception of the fundamental work of Emil Fischer in the last two decades of the past century, the chemistry of carbohydrates is in an amazing state of development. Chemists of all countries take part in this work. The papers are scattered through a great number of journals dealing with scientific and industrial chemistry in nearly every language. The reason for this is the versatility and variability of the carbohydrates and their importance in many chemical fields. This field of chemistry is extremely rich in stereochemical problems as well as on those of tautomerism.

The carbohydrates are surpassed in this regard only by the proteins. Any end to the growth of carbohydrate chemistry cannot be perceived at this time. This branch of chemistry extends from very simple compounds of low molecular weight to substances of high molecular weight. It is also closely connected with other types of natural compounds, such as proteins, nucleins, and others.

Therefore, the book of Pigman and Goepp meets an urgent requirement of all hemists interested in every aspect of carbohydrates. It is the most recent, modern, p-to-date book in this field, and as far as I know, the only one written in English. Whoever wishes to study or teach carbohydrate chemistry for chemical, industrial, edical or biological reasons, advanced students as well as mature scientists, will find this book a very good starting point and, in many cases, a very complete compre- nsion for his information and further work. Through a great number of bibliographic ta the search of the original literature is facilitated.

The great number of facts presented clearly and systematically is astonishing.

The writing of this book took about 8 years, the authors state in their acknowledgement, and thank several persons for assistance and advice. Among those thanked are to be found such well known names as C. S. Hudson, H. S. Isbell, D. H. Brauns and others. Their help is a proof too of the high standard of the book.

The scientific achievement of the authors will add to the further growth of carbohydrate chemistry, an indispensable help for the theoretical and practical worker in this field.

B. HELFERICH, Bonn, Germany

Erratum

Volume 23, Number 1, p. 78

The first sentence under Discussion should read as follows: The horegoing experiments demonstrate that 1-glyceraldehyde inhibits the fexokinase activity of mammalian tissues.

Rutin Content of Several Varieties of *Nicotiana rustica* and *N. glauca* [1]

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Introduction

In connection with research being conducted at this Laboratory in an attempt to find materials, other than nicotine, of economic importance in *Nicotiana rustica*, investigation of the rutin content was made. Rutin is obtained commercially from buckwheat (1,2) and is important in medicine for control of capillary fragility (3,4) and certain other hemorrhagic conditions. The rutin contents of several species of *Nicotiana* have been reported (5–9).

The present work was confined to *N. rustica*, varieties Olson 68, Armenia, Prosecknskaia (local), Slepukhinskaia (local), Stalingradskaia and Indian (United Provinces) and to a few analyses of *N. glauca*.

Experimental

The plants were grown on the Laboratory grounds.[3] The whole rustica plant was cut and, within one hour after cutting, the leaf web was stripped from the midrib. The leaf web was then cut into approximately one-inch squares with a knife. A sufficient number of plants was harvested to obtain 1000–1500 g. of this material. It was tossed and tumbled in a large metal container to insure adequate mixing before sampling. Four samples were taken simultaneously, two of approximately175 g. each for duplicate rutin analyses and two of approximaetly 350 g. each for duplicate moisture and nicotine analyses. This technique minimizes sampling error (10) as concordant analytical results on duplicate samples could be realized in this manner. This was not true of any other sampling techniques tried.

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[1] Report of a study made under the Research and Marketing Act of 1946.

[2] One of the Laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

[3] Olson 68 is a standard American variety. The four Russian varieties were obtanied through the Russian Embassy; the Indian variety was obtained from the Indian Department of Agriculture.

The plants were analyzed at frequent intervals throughout the growing season. All analyses were made on fresh green leaves of the healthy plant, unless otherwise noted. The method for determining rutin was based on work by Naghski, *et al.* (11), of this Laboratory. The fresh material was extracted with acetone, and the resulting extracts were concentrated until free of acetone. The residue was boiled with sufficient water to dissolve the rutin, and the water-insoluble fats and pigments were removed by filtration. The insoluble residue and filter paper were again extracted with a small quantity of boiling water and filtered. The combined filtrates were stored at room temperature overnight and then in a refrigerator until precipitation of the rutin was complete (from 1 to 7 days, depending on the quantity). The precipitate was recovered on a tared Gooch crucible, dried at 110°, and calculated as percent crude rutin. Rutin prepared in this manner was approximately 85% pure (11). Moisture was determined by drying at 110°. Most of the nicotine analyses were made by an ultraviolet absorption method (12), but some were made by silicotungstic acid precipitation (13). Results obtained by the two methods agreed.

Results and Discussion

The results of the analyses are summarized in Table I. Table II gives the maximum weights of leaves, of nicotine, and of rutin per plant.

For each variety, there is evidence of two maxima in respect to rutin content. These maxima occurred 11 to 30 days apart, and since the varieties were planted within 3 days of each other, the maxima would seem to result from the natural physiological functions of the plant. Although there was a dry period in the growing season necessitating irrigation, the minima did not correspond to this period.

From previous investigation on *N. rustica*, it was known that nicotine content per plant increases steadily to maturity. Fig. 1 shows how rutin, nicotine, and plant weight vary with the age of the plant, using the data of Olson 68 as an example. Although absolute values for the other varieties studied are different, the trends shown here are representative of all. In general, the date at which the maximum nicotine content of a given variety was reached, coincided with the date of maximum weight of leaves per plant to 6 days thereafter. Maximum rutin occurred between the leaf weight maximum and nicotine maximum. Since this rutin maximum was sharp, the selection of the harvest date would be critical, if it is desired to obtain both rutin and nicotine from the plant.

Olson 68 was superior to all other rustica varieties studied in respect to rutin, nicotine, and leaf weight per plant. *Nicotiana glauca*, however, was far superior to rustica in rutin content. Samples of *N. glauca* analyzed 75, 87, and 94 days after transplanting contained 1.18, 1.34,

and 1.88% rutin, respectively, on a moisture-free basis. A sample of suckers (lateral shoots) taken 94 days after transplanting contained 2.08% rutin. The *N. glauca* was sampled by stripping about one-third of the mature lower leaves from each plant each time analyses were

TABLE I

Weight of Leaves and Rutin and Nicotine Content of Nicotiana rustica *at Various Stages of Growth*

Days from transplanting	Weight of leaves per plant[a b]	Rutin content[a]	Nicotine content[a]	Weight of rutin per plant[b]	Weight of nicotine per plant[b]
Olson 68					
	g.	%	%	g.	g.
5	0.3	0.47	—	<0.01	—
26	5.1	0.51	1.05	0.03	0.05
39	10.6	0.66	1.49	0.07	0.16
49	27.7	0.59	2.00	0.16	0.55
63	55.1	0.52	3.16	0.29	1.74
77	70.8	0.42	5.98	0.30	4.23
85	105.2	0.22	5.97	0.23	6.28
91	106.3	0.43	7.44	0.46	7.90
98	84.2	0.23	9.48	0.19	7.98
105	71.2	trace	8.93	trace	6.36
Armenia					
40	20.8	0.82	1.75	0.17	0.36
66	50.6	0.89	5.29	0.45	2.68
73	64.5	0.56	5.81	0.36	3.75
80	65.9	0.47	7.04	0.31	4.64
88	67.3	0.58	6.58	0.39	4.43
94	58.0	0.54	5.66	0.31	3.28
Proseckneskaia (local)					
38	20.1	0.73	2.35	0.15	0.47
66	59.7	0.55	5.70	0.33	3.40
73	78.9	0.37	5.63	0.30	4.23
80	65.9	0.37	5.57	0.30	4.57
88	64.0	0.31	5.88	0.20	3.76
94	63.4	none	5.97	none	3.78

TABLE I *Continued*

Days from transplanting	Weight of leaves per plant[a] [b]	Rutin content[a]	Nicotine content[a]	Weight of rutin per plant[b]	Weight of nicotine per plant[b]
		Slepukhinskaia (local)			
	g.	%	%	g.	g.
38	25.5	0.70	2.27	0.18	0.58
68	62.5	0.39	5.47	0.25	3.42
74	72.9	0.33	5.61	0.24	4.09
82	80.6	0.44	4.28	0.36	3.45
88	72.8	0.31	5.45	0.23	3.97
94	74.5	trace	5.25	trace	3.91
		Stalingradskaia			
38	20.6	0.72	1.97	0.15	0.40
68	52.4	0.37	5.55	0.19	2.91
74	68.0	0.31	4.98	0.21	3.38
82	73.4	0.36	4.67	0.27	3.43
89	72.0	0.40	4.90	0.29	3.53
94	68.2	0.12	4.64	0.08	3.16
		Indian (United Provinces)			
51	37.7	0.46	0.76	0.17	0.29
65	43.6	0.58	0.34	0.25	1.02
71	58.7	0.31	3.12	0.18	1.84
79	67.7	0.44	5.05	0.30	3.42
86	63.5	0.44	6.52	0.28	4.14
93	45.9	0.31	5.97	0.14	2.74

[a] Moisture-free basis.

[b] "Per plant" refers to leaf web only exclusive of mid-rib, petiole, or stalk of the plant. Although there may be nicotine and rutin in these other portions of the plant, analyses were not made on them.

made. This did not permit the weight per plant to be determined for this species. Suckers and flower heads of the rustica varieties, examined separately, contained an average of 0.4% rutin 38 to 42 days after transplanting. A sample of frenched, virus-infected leaves of Olson 68 taken at 86 days contained 0.45% rutin, which is about double the amount found in the normal plant at this age. However, since the diseased plants were about one-half the size of the normal plant, the quantity of rutin per plant was about the same as that of the normal.

TABLE II

Age at which Maximum Weights of Leaves, Rutin, and Nicotine were Attained in Six Varieties of Nicotiana rustica

Variety	Max. weight of leaves per plant		Max. weight of nicotine per plant		First max. weight of rutin per plant		Second max. weight of rutin per plant	
	g.	*age, days*	*g.*	*age, days*	*g.*	*age, days*	*g.*	*age, days*
Olson 68	108	88	8.2	94	0.31	72	0.46	91
Armenia	67	85	4.8	83	0.46	67	0.39	88
Prosecknskaia	82	80	4.6	79	0.33	65	0.30	80
Slepukhinskaia	81	83	4.2	78	0.26	55	0.36	82
Stalingradskaia	73	82	3.5	85	0.18	58	0.30	88
Indian (United Provinces)	68	80	4.2	85	0.25	65	0.31	81

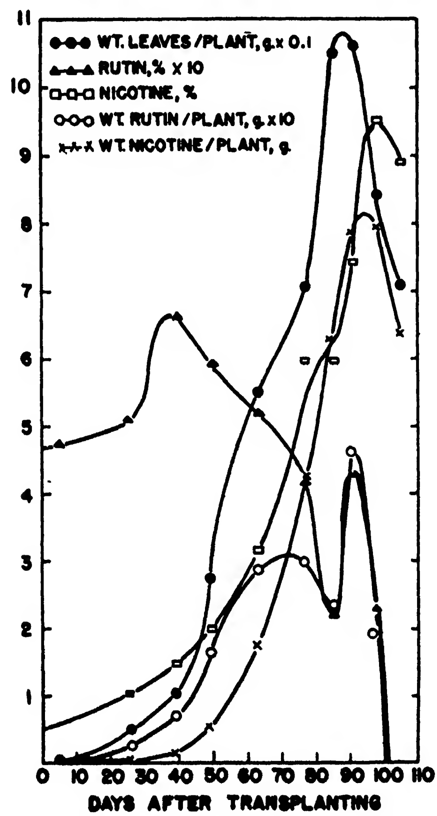

FIG. 1. Weight of leaves and rutin and nicotine contents of *Nicotiana rustica*, variety Olson 68, at various stages of growth. (Moisture-free basis.)

Acknowledgment

The authors express their thanks to C. O. Willits, J. Naghski and C. Ricciuti for helpful suggestions and for the moisture analyses.

Summary

Rutin was found in six varieties of *Nicotiana rustica* not previously reported. The nicotine content attained a single maximum percentage within 6 days of maxiumm leaf weight. Although there is some evidence that the rutin content characteristically showed two peaks, from 11 to 30 days apart, the second and highest occurring between leaf weight and nicotine maxima, the quantity of samples employed might not lead to a valid conclusion. The variety *Prosecknskaia*, for instance, did not show two rutin peaks. Larger amounts of rutin (up to 2%) were found in *Nicotiana glauca* than have been reported previously.

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The Role of Choline Oxidase in Labilizing Choline Methyl[1,2]

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Introduction

Anearobically, methylation of homocysteine in the presence of choline in rat liver homogenates is small or negligible; aerobically, the rate of methylation approaches that from betaine (1). Since choline is rapidly oxidized to betaine in the presence of oxygen under these conditions (2,3,4,5), it seemed likely that choline oxidase played an important role in making choline methyl available for transmethylation. It was found that the liver and kidney of animals lacking an active choline oxidase were unable to methylate homocysteine directly *in vitro*. In animals with choline oxidase the amount of methionine formation from choline paralleled the activity of choline oxidase under varying experimental conditions.

Methods

Livers from animals killed by stunning were homogenized with 2 parts of 0.015 *M* potassium phosphate buffer at pH 7.4 in the homogenizer of Potter and Elvehjem (6) and strained through cheese cloth.

DL-Homocysteine was prepared from DL-methionine by the method of Stekol (7). Choline chloride and betaine hydrochloride were Merck reagent grade.

Four milliliters of solution containing 1 ml. of 1:2 liver homogenate and the substrates were incubated in the apparatus previously described (8). After incubation the solutions were deproteinized by the addition of 1 ml. of 20% trichloroacetic acid and filtered. Guinea pig liver solutions were brought to a boil before filtering.

Methionine was determined by a modification of the method of McCarthy and Sullivan (9). The procedure was a follows: 0.2 ml. of 5 *N* NaOH and 0.1 ml. of 1% freshly prepared sodium nitroprusside were added to 2 ml. of the trichloroacetic acid filtrate and allowed to stand 15 min. at room temperature. One milliliter of 85% phosphoric acid was added, and after 15 min. the solutions were read in a Klett-Summerson colorimeter with a green filter. All values reported were carried out in duplicate or triplicate. The experiments presented are typical.

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[1] A preliminary report of this work appeared in the *Federation Proc.* **8**, 195 (1949).

[2] Aided by a grant from the U. S. Department of Health.

Results

Bernheim and Bernheim (5) were unable to demonstrate the presence of choline oxidase in the liver and kidney of guinea pigs, and we have shown that this enzyme is relatively inactive in liver and kidney homogenates of rabbits. These two animals show no methylation of homocysteine by choline in liver homogenates although betaine is active under similar conditions (Table I).

TABLE I

Methylation of Homocysteine in Animals Lacking an Active Choline Oxidase

Substrate	Guinea pig		Rabbit	
	O_2	N_2	O_2	N_2
DL-homocysteine	1.0	0.7	1.9	2.6
+choline	+0.0	−0.2	+0.1	+0.1
+betaine	+1.4	+1.8	+2.8	+2.8
+dimethylthetin	+6.4	+6.6	+7.6	+7.4

Substrate concentrations: 15 mg. %. Total volume: 4 ml. containing 1 ml. of 1:2 liver homogenate in pH 7.5 potassium phosphate buffer. Time: 2 hr. Figures are mg. % methionine. All values reported in these tables are the average of duplicates or triplicates. Probable error ± 0.15 mg. %.

Choline is active in methionine formation under aerobic conditions in liver homogenates of all animals having an active choline oxidase. Typical experiments are shown in Table II. Four per cent oxygen was used instead of air for the aerobic experiments to minimize the oxidation of homocysteine. Since this also limits the oxidation of choline in the mouse, it was necessary to incubate the choline and mouse liver homogenate in air to permit adequate choline oxidation. Homocysteine was then added and the experiment continued in 4% oxygen. The amount of methionine formed under these conditions equaled the methionine formation from betaine under similar conditions whereas without the preliminary incubation of choline in air very little methionine was formed from choline in this animal under 4% oxygen.

In earlier experiments using liver slices (1) methionine formation from betaine was decreased in the presence of oxygen. This was ascribed to the oxidation of homocysteine. On the other hand methionine formation from choline was only slightly affected by oxygen. The

action of oxygen in depressing the homocysteine concentration was balanced by its action in converting choline into betaine which is the more effective compound in this reaction. Since choline was active even under anaerobic conditions, it was assumed that oxidation was not an obligatory step in this reaction. However, under the experimental conditions employed a certain amount of oxidation of choline could have occurred before the oxygen was completely swept out of the

TABLE II

Methionine Formation in Liver Homogenates of Animals Having an Active Choline Oxidase

Animal	Substrate	Nitrogen		4% oxygen	
		Total	Net increase	Total	Net increase
Chick (10)	Homocysteine	4.0		4.4	
	Homocysteine+choline	4.0	+0.0	4.3	−0.1
	Homocysteine+betaine	1.1	+1.1	5.2	+1.8
Chick (10)	Homocysteine	2.6		1.7	
	Homocysteine+choline	2.6	+0.0	2.3	+0.6
	Homocysteine+betaine	4.4	+1.8	3.8	+2.1
Mouse (11)	Homocysteine	1.8		1.6	
	Homocysteine+choline	2.1	+0.3	2.8	+1.2
	Homocysteine+betaine	3.7	+1.7	2.8	+1.2
Rat (3,4,5) (Av. of 12 animals)	Homocysteine+choline		+0.26±0.15		*range* +2 *to* +6

Conditions as in Table I. In the case of the aerobic mouse experiment the choline was incubated for 1 hr. in air before adding homocysteine. References to demonstration of choline oxidase shown in brackets.

vessels. In the present studies experiments were designed to maintain complete anaerobiosis to test this point. The tray containing the reaction vessels was placed in the water bath (8) which had been cooled with finely chipped ice. Substrate solutions and degassed liver homogenate were added. The tray cover was replaced, and the apparatus flushed with nitrogen which had been passed over hot reduced copper. After 30 min. the bath was brought to 38°C. and the experiment con-

tinued with a slow flow of nitrogen. Even with these precautions choline was slightly active (Table III). It is unlikely that any appreciable amounts of choline could be oxidized at the low temperature during the period of gassing or that any appreciable amounts of oxygen were present during the incubation period. Somewhat lower values were also obtained when evacuated and flushed Warburg vessels were used. The differences in the two types of experiments may possibly be ascribed to the fact that choline is continuously present in one case and tipped into the medium after a period of time in the other. While the possibility remains that the positive values found in about two-thirds of the experiments were due to technical difficulties in removing the last traces of

TABLE III

Anaerobic Methionine Formation in Rat Liver Homogenates

Sex	Blank	+Homocysteine	+Homocysteine + choline	Net increase
♀	2.1	3.4	3.6	+0.2
♀	1.5	2.8	3.2	+0.4
♂	1.6	4.4	5.0	+0.6
♂	1.5	4.6	5.2	+0.6

All stock solutions evacuated and cooled before adding to reaction beakers. The beakers were shaken for 30 min. at 3°C. under a stream of nitrogen passed over hot copper. The temperature was then raised to 38°C., and the experiment continued for 3 hr. Final concentrations 15 mg. % in 0.015 phosphate buffer of pH 7.6. Figures are mg. % of methionine in filtrates.

oxygen, it seems more likely that there is a true but very variable anaerobic effect. Its rate is relatively insignificant ($Q = 0.01$)[2] compared with aerobic choline oxidation to betaine ($Q = 0.3$) and methionine formation from betaine ($Q = 0.1$). However, even this small anaerobic methionine formation in the rat cannot be considered evidence for a direct methylation of homocysteine. Welch and Irving have shown that choline oxidase behaves like a typical dehydrogenase (2) and Mann, Woodward, and Quastel (12) have shown that ferricyanide can effectively replace oxygen in choline oxidation. It is, therefore, probable that some anaerobic oxidation of choline to betaine can occur through the medium of naturally occurring hydrogen acceptors

[2] Q is the amount of substance formed or utilized computed as a gas in µl./mg. dry weight of tissue/hr.

and so account for the anaerobic methionine formation. Regardless of the mechanism involved it can be shown by tracer techniques that sufficient betaine is formed from choline anaerobically under our experimental conditions to account for all the methionine formed in the presence of choline (Table IV). A more detailed discussion of anaerobic oxidation and more complete data will be presented at a latter date.

TABLE IV

Formation of Betaine from C_{14} Methyl Labeled Choline in Rat Liver Homogenates

	Expt. 1	Expt. 2
Total counts in choline added	6.3×10^4	11.2×10^4
Total counts in betaine found	0.22×10^4	1.20×10^4
Millimols betaine found	0.0047	0.010
Millimols methionine formed from choline and homocysteine in parallel experiment	0.0013	0.0033

All figures are per 100 ml. solution. Condition as in previous experiments. Vessels were evacuated and flushed 5 times with purified N_2 before choline was added. Choline and betaine isolated as Reinecke salts after the addition of 20 mg. carrier.

Bernheim and Bernheim (36) have shown that choline oxidase is present in the washed dialyzed residue of a rat liver homogenate.[3] Such a preparation is unable to utilize betaine or choline for methionine formation. The supernatant liquid can utilize betaine but not choline (Table V). The residue containing choline oxidase and oxygen is necessary for an effective utilization of choline methyl by the supernatant liquid. This evidence for the need for two liver fractions for choline utilization in methionine synthesis cannot be reconciled with the concept of a simple direct transfer of choline methyl to homocysteine.

At pH 7.8 choline is oxidized via betaine aldehyde to betaine. The order of effectiveness of these compounds in methionine formation at this pH is in accord with the concept that oxidation of choline to betaine increases the lability of choline methyl (Table VI). At pH 6.6 choline is oxidized to betaine aldehyde, but betaine formation is almost completely inhibited (5). Under these conditions only betaine is effective in methionine formation.

[3] The statement that choline is ineffective as a methyl donor in dialyzed liver preparations (1) was based on experiments done on lyophilized liver. Dialysis of whole liver homogenates does not decrease but may enhance the choline effect.

TABLE V

Fractionation of Whole Homogenate for Methionine Formation

	Supernatant		Sediment		Supernatant + sediment	
	Total	Net increase	Total	Net increase	Total	Net increase
Air						
Blank	1.8		1.4		3.2	
+DL-homocysteine	4.6		1.5		4.6	
+DL-homocysteine+choline	4.5	−0.1	1.6	+0.1	6.8	+2.2
+DL-homocysteine+betaine	7.5	+2.9	1.3	−0.2	7.5	+2.9
Nitrogen						
Blank	2.1		1.5		2.3	
+DL-homocysteine	3.9		1.6		4.6	
+DL-homocysteine+choline	3.8	−0.1	1.5	−0.1	4.7	+0.1
+DL-homocysteine+betaine	6.2	+2.3	1.7	+0.2	6.8	+2.2

One milliliter of 1:2 rat liver homogenate in 0.015 *M* phosphate buffer at pH 7.5 centrifuged at 2000 × g. Total volume: 4 ml. Concentrations: 12.5 mg. %. Figures are mg. % methionine ± 0.15 mg. %. All solutions were incubated in air or 100% nitrogen for 1.5 hr. without homocysteine. The homocysteine was then added, and the experiment continued under nitrogen in both cases.

TABLE VI

pH Effect on Methionine Formation

Substrate added	Methionine in mg. %			
	pH 7.8		pH 6.5	
	Total methionine	Increase over homocysteine alone	Total methionine	Increase over homocysteine alone
DL-homocysteine	3.1		2.0	
DL-homocysteine+choline	3.9	+0.8	2.0	+0.0
DL-homocysteine+betaine aldehyde	4.3	+1.2	2.2	+0.2
DL-homocysteine+betaine	5.6	+2.5	4.2	+2.2

1:2 rat liver homogenate in 0.05 *M* potassium phosphate, pH as indicated. 4% O_2 in N_2. 2 hr. Substrates 20 mg. %. 38°C.

Of the compounds related to choline in structure shown in Table VII only sulfocholine is active (13). This compound increases the oxygen consumption of liver homogenates (14). The oxidation of the hydroxyl should give rise to dimethylthetin which is the most active methyl donor yet found (15). Sulfocholine in large amounts is toxic in the

TABLE VII

Choline Analogues in Methionine Formation

Substrate	Total methionine	Less tissue	Less homo-cysteine
	mg.%		
	2.6		
DL-homocysteine	4.7	2.1	
homocysteine+choline	5.7	3.1	+1.0
homocysteine+betaine	6.9	4.3	+2.2
homocysteine+arsenocholine	4.8	2.2	+0.1
homocysteine+phosphocholine	4.5	1.9	−0.2
homocysteine+Ca phosphorylcholine	4.9	2.3	+0.2
homocysteine+S choline	8.2	5.6	+3.5
homocysteine+choline+arsenocholine	6.1	3.5	+1.4
homocysteine+choline+phosphocholine	5.0	2.4	+0.3
homocysteine+Ca phosphorylcholine	5.9	3.3	+1.2

All concentrations 25 mg. % in 4 ml. pH 7.5 phosphate buffer containing 1 ml. of 1:2 rat liver homogenate. Gas: 4% O_2 in N_2. Time 2 hr. Probable error: ± 0.15 mg. %.

whole animal (16). Arsenocholine (12) and phosphocholine also in-increase the oxygen uptake of liver homogenates; the expected products arsenobetaine and phosphobetaine, respectively, are inactive (Table VIII). Of the choline analogues tested only phosphocholine inhibits methionine formation from choline in rat liver. It can be seen from Fig. 1 that phosphocholine also inhibits the oxidation of choline by choline oxidase. Arsenocholine does not inhibit choline oxidation (12) and does not inhibit methionine formation from choline or betaine (Table VIII).

That the labilization of the methyl in betaine is not simply due to the strongly negative carboxyl is suggested by the inactivity of calcium

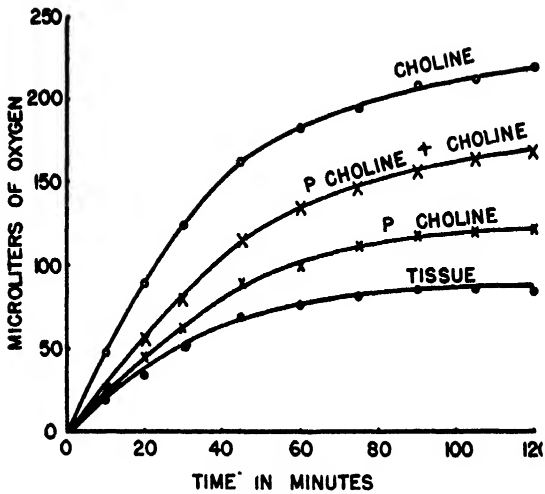

FIG. 1. Inhibition of choline oxidation by phosphocholine.

TABLE VIII

Betaine Analogues in Transmethylation

Substrate	Total methionine *mg. %*	Net increase *mg. %*
Homocysteine	5.2	
Homocysteine+choline	6.2	1.0
Homocysteine+betaine	7.0	1.8
Homocysteine+phosphobetaine	5.3	0.1
Homocysteine+arsenobetaine	5.3	0.1
Homocysteine+serinebetaine	5.1	−0.1
Homocysteine+alanylbetaine	5.1	−0.1
Homocysteine+choline+phosphobetaine	6.1	0.9
Homocysteine+choline+arsenobetaine	6.3	1.1
Homocysteine+choline+serinebetaine	6.1	0.9
Homocysteine+choline+alanylbetaine	6.2	1.0
Homocysteine+betaine+phosphobetaine	7.0	1.8
Homocysteine+betaine+arsenobetaine	7.1	1.9
Homocysteine+betaine+serinebetaine	6.8	1.7
Homocysteine+betaine+alanylbetaine	6.8	1.7

1:2 rat liver homogenate. 4% O_2. 3 hr. Probable error: ± 0.1 mg. %.

phosphorylcholine which like betaine has a negative radical opposite the quaternary nitrogen. Calcium phosphorylcholine is active in promoting growth with homocysteine in the whole animal (17). This difference between the *in vivo* and *in vitro* experiments may be explained by the

TABLE IX

Effect of the Nitrogen Mustard bis(beta Chloroethyl) Methylamine Hydrochloride

Homocysteine	Choline	Betaine	Poison	mg. % Methionine	Net increase
−	−	−	−	2.9	
+	−	−	−	4.4	
+	+	−	−	5.0	+0.6
+	+	−	+	4.2	+0.2
+	−	+	−	5.6	+1.2
+	−	+	+	5.5	+1.1
−	−	−	+	2.7	−0.2

Conc. of nitrogen mustard 10^{-4} *M*. Other conditions as in Table I. 96% N_2 4% O_2.

fact that this compound is not hydrolyzed to choline in the rat liver (18) but is hydrolyzed in the whole animal (19).

The nitrogen mustard bis (β-chloroethyl) methylamine inhibits choline oxidase at very low concentrations (20). It effectively prevents methionine formation from choline (Table IX). Sodium azide and potassium ferricyanide inhibit choline oxidase but also interfere with the determination of methionine.

Discussion

While other pathways for choline methyl cannot be excluded, there is no evidence for a direct reaction with homocysteine. In the light of these *in vitro* findings a reinterpretation of the data from whole animal experiments which have been considered to be evidence for a direct methylation is indicated. Rat growth experiments with homocystine on methionine deficient diets suggested that choline was more effective than betaine as a methyl donor (22). In view of the multiple roles of choline in metabolism (24), this conclusion does not seem justified for not only methionine but also choline must be synthesized in these methyl deficient animals. Choline can be used directly for phospholipide formation, and it may be rapidly oxidized to betaine for methionine synthesis as shown here. This reaction is not directly reversible (25,26) but, according to present knowledge, betaine must go through at least four steps to form choline (24). Since only one of the three methyls of betaine appears to be labile (1,27), three mols of betaine are required per mol of choline synthesized (28,29). It is clear that the

overall efficiency of betaine may be less than that of choline for needs of the methyl deficient rats even though the present data showed that betaine methyl is more directly available for methionine synthesis. Where the minimum needs of the animal for choline have been satisfied, betaine may be more effective for growth than choline (30). Such growth experiments cannot give evidence for the mechanism of choline utilization in methionine synthesis.

On the basis of the finding that the rate of synthesis and the total amount of choline is normal in hemorrhagic kidneys of rats on a choline deficient diet Jacobi and Bauman (31) concluded that the effect of choline on kidney lesions involves the methyl-releasing action of choline oxidase, and that the inhibition of choline oxidase by the fatty acids which have accumulated prevents the formation from choline of an unknown "methyl factor" (not methionine). A simpler interpretation of choline activity based on its lipotropic effect seems more likely because arsenocholine (32) or triethylcholine (33) prevents the condition more efficiently than labile-methyl-containing compounds as betaine and methionine. The inhibition of choline oxidase by fatty acids (34) rather than aggravating the lesions may increase the choline concentration by diminishing the oxidation of choline to betaine. This may in part account for the observed spontaneous regression of kidney lesions. The delayed recovery in this condition and the lag in the betaine effect in the growth experiments with homocysteine (22) may also reflect the slow readjustment of the dynamic steady state and its adaptation to the abnormal necessity of meeting all the choline requirements by synthesis.

Although methionine formation from choline could not be demonstrated *in vitro* in guinea pig homogenates, methyl labeled methionine has been isolated from the tissues of guinea pigs fed C_{14} methyl-labeled choline (21). The recent isolation of labeled betaine from these animals (14) demonstrates that a mechanism for the conversion of choline to betaine must exist in the guinea pig even though it has not been shown *in vitro*. The isolation of labeled betaine brings the *in vivo* guinea pig data into agreement with the postulate that betaine is an intermediary in choline methyl utilization.

The complete correlation between the oxidation of choline and its activity in methionine formation in these experiments supports the concept that the oxidation of the alcohol group of choline is necessary for the labilization of the methyl group. This establishes the role of

choline oxidase in the organism and betaine as an intermediate in choline methyl utilization. In this view dimethylglycine not dimethylethanolamine is the end product of choline demethylation.

An active choline oxidase should make the animal more independent of the labile methyl composition of the diet by permitting a diversion of choline methyl to methionine synthesis, but such an animal is more susceptible to choline deficiency. The relative inactivity of choline oxidase in the guinea pig liver makes choline methyl less readily available, and therefore, this animal is less likely to be drained of choline by the presence of methyl acceptors in the diet (35).

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Summary

1. Methylation of homocysteine in the presence of betaine is relatively independent of the oxygen tension.

2. Methylation of homocysteine in the presence of choline approaches that from betaine aerobically but is small or negligible anaerobically.

3. The degree of utilization of choline methyl for methionine synthesis parallels the activity of choline oxidase.

4. No methionine formation has been observed in liver homogenates of animals which do not have an active choline oxidase.

5. Conditions of pH, anaerobiosis, and inhibitors which decrease the oxidation of choline to betaine decrease methionine formation from choline.

6. The effect of choline and betaine in whole animal experiments is reconsidered in the light of these findings. No evidence for a direct methylation of homocysteine has been presented.

7. It is concluded that choline methyl is not transferable to homocysteine unless the alcohol group has been oxidized.

8. Choline oxidase is considered to regulate the extent of choline methyl utilization.

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The Purine and Pyrimidine Requirements of *Leuconostoc citrovorum* 8081 [1]

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Nutrition of Leuc. citrovorum

Recently Shive *et al.* (1) demonstrated a relationship between thymidine, vitamin B_{12} concentrate, and purines in the nutrition of *Lactobacillus lactis* Dorner. They observed that thymidine and guanylic acid or thymidine, guanine, and hypoxanthine could replace vitamin B_{12} in the growth of this organism. Furthermore, vitamin B_{12} concentrates could replace both purines and thymidine. Moreover, Shive *et al.* (1) have reported that thymidine, after a lag phase, slowly replaced both the folic acid and the vitamin B_{12} required for the growth of *Lactobacillus leichmannii*.

The factor required for the growth of *Leuconostoc citrovorum* 8081 (2) appears to be similar in function to that of vitamin B_{12} to the extent that it can replace thymidine and folic acid (2,3). Therefore, in an attempt to study the function of the unknown factor and to further differentiate it from that of thymidine or vitamin B_{12}, an investigation was made of the purine and pyrimidine metabolism of *Leuc. citrovorum*.

Methods

The procedures and techniques employed were similar to those previously reported (2,4).

Leuc. citrovorum was grown on the basal medium complete except for the variations noted when purine and pyrimidine requirements were being studied. A source of the growth factor required by *Leuc. citrovorum* was supplied by the addition of controlled amounts of Reticulogen-360 (Parenteral Liver Extract, Eli Lilly and Company) or of

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a very active liver concentrate (No. 1) (1 citrovorum unit = 0.09 μg.) (4). In a few experiments a relatively active rat urine concentrate (No. 2) (1 citrovorum unit = 0.2 μg.) (4) was used.

Results

When adenine, guanine, xanthine, and uracil were omitted from the basal medium, the growth responses of *Leuc. citrovorum* to graded amounts of the essential growth factor furnished by Reticulogen, liver concentrate No. 1, or rat urine concentrate No. 2 were greatly reduced (Table I). The growth responses of the organism to thymidine and to

TABLE I

Effect of Purines and Pyrimidines upon the Activity of "Citrovorum" Preparations in Stimulating the Growth of Leuconostoc citrovorum 8081

Liver concentrate No. 2 μg.	Purines and pyrimidines[a]	*G* Value[b]
0	+	97
0	−	97
1.16	+	57
2.32	+	34
3.48	+	27
1.16	−	91
2.32	−	85
3.48	−	81
5.80	−	77
11.60	−	63

[a] Adenine, guanine, uracil, and xanthine were added at a level of 100 μg. of each per 10 ml. assay tube where indicated.

[b] An 18 hr. turbidimetric assay procedure was used (660 mμ. filter of the Evelyn colorimeter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay). All amounts given above are totals per 10 ml. assay tube.

high amounts of folic acid were completely eliminated when the purines and pyrimidines were omitted from the culture medium. This indicates that certain of the purines or pyrimidines are essential for the growth of *Leuc. citrovorum*. However, some growth was noted in the absence of purines and pyrimidines when increased amounts of Reticulogen, liver concentrate, or rat urine concentrate were used (Table I). This observation would also indicate that the concentrates were contributing measurable amounts of the essential purines or pyrimidines, or that they contained a compound capable of replacing or synthesizing the required purines or pyrimidines.

Upon further investigation, it was found that xanthine, hypoxanthine and guanine were active in stimulating the growth of *Leuc. citrovorum* whereas adenine, uracil, and thymine were inactive (Table II). At a level of 10 μg./ml. of culture medium, xanthine and hypoxanthine were approximately equal in activity; guanine was slightly less active; combinations of xanthine, hypoxanthine, and guanine were more active than any one of the three alone (Table II). When tested at a level of 20 μg./ml. of culture medium, xanthine was somewhat more active than hypoxanthine or guanine (Table III); xanthine alone or a combination of guanine and hypoxanthine were capable of furnishing the

TABLE II

Effect of Purines upon the Growth of Leuconostoc citrovorum

Variable components of the culture medium[a]			G Value
Xanthine	Hypoxanthine	Guanine	
$\mu g.$	$\mu g.$	$\mu g.$	
0	0	0	91
100	0	0	49
0	100	0	49
0	0	100	56
100	100	100	40
100	100	0	40
0	100	100	41
100	0	100	41

[a] Basal medium used with the purines and pyrimidines omitted except as noted above. All amounts given above are totals per 10 ml. assay tube. Each assay tube contained 2.32 μg. of the liver concentrate (see text). An 18 hr. turbidimetric assay procedure was employed (660 mμ. filter of the Evelyn colorimeter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay).

entire purine requirement of *Leuc. citrovorum* (Table II and III). Usually guanine or hypoxanthine alone would not permit quite maximal growth under the conditions employed. When the organism was grown on suboptimal amounts of either xanthine, guanine, or hypoxanthine, the addition of adenine, uracil, and thymine had a sparing effect (Table III). The effect was found to be due primarily to the adenine, although adenine alone could not fulfill the purine requirements of the organism.

Guanosine was approximately equal to guanine on a molar basis, but

TABLE III

Purine and Pyrimidine Requirements of Leuconostoc citrovorum

Variable components of the culture medium[a]				G Value
Xanthine	Hypoxanthine	Guanine	Adenine	
μg.	μg.	μg.	μg.	
0	0	0	0	90
100	100	100	100	34
5	0	0	0	85
25	0	0	0	75
100	0	0	0	48
200	0	0	0	35
0	0	0	100	90
5	0	0	100	71
15	0	0	100	58
0	5	0	0	85
0	25	0	0	75
0	100	0	0	48
0	200	0	0	42
0	5	0	100	84
0	15	0	100	72
0	0	5	0	88
0	0	25	0	80
0	0	100	0	51
0	0	200	0	48
0	0	5	100	82
0	0	15	100	57
5	0	5	100	60
5	0	5	0	84

[a] Basal medium used with the purines and pyrimidines omitted except as noted above. All amounts given are totals per 10 ml. assay tube. Each assay tube contained 2.32 μg. of the liver concentrate (see text). An 18 hr. turbidimetric assay procedure was employed (660 mμ. filter of the Evelyn colorimeter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay).

guanylic acid was less active (Table IV). Cytidylic acid, uric acid, allantoin, benzimidazole, caffeine, theophylline, 6-methylthiouracil, and high levels of pantothenic acid or of *p*-aminobenzoic acid were ineffective in replacing the purine requirement of *Leuc. citrovorum*. None of these compounds, except benzimidazole, were inhibitory towards the organism when grown in suboptimal amounts of xanthine, guanine, or hypoxanthine. Benzimidazole showed some inhibition

against *Leuc. citrovorum* when the organism was grown in a medium containing suboptimal amounts of hypoxanthine but not when grown on limiting amounts of xanthine or guanine (Table IV). Woolley (5) has reported that benzimidazole inhibited the growth of *Streptococcus lactis* and *Escherichia coli*, and that guanine and adenine overcame its action on *E. coli* and uracil removed the antagonism toward *S. lactis R.*

Similarly, folic acid and thymidine were unable to replace the purines required by *Leuc. citrovorum*. The ability of high levels of folic acid to

TABLE IV

Effect of Purines, Pyrimidines, and Their Derivatives upon the Growth of Leuconostoc citrovorum

Purines or pyrimidines added[a]	Amount	Benzimidazole added	G Value
	$\mu g.$	$\mu g.$	
None	—	0	90
Cytidylic acid	(200–1000)	0	90
Guanylic acid	100	0	84
Guanylic acid	500	0	75
Guanosine	50	0	80
Guanosine	100	0	64
Guanosine	500	0	55
Guanine	50	0	69
Guanine	250	0	48
None	—	2000	95
Guanine	50	1600	69
Xanthine	50	0	69
Xanthine	50	1600	68
Hypoxanthine	50	0	65
Hypoxanthine	50	400	79
Hypoxanthine	50	1600	88

[a] Basal medium used with the purines and pyrimidines omitted except as noted above. All amounts given above are totals per 10 ml. volume assay tube. An 18 hr. turbidimetric assay procedure was employed (660 mμ. filter of the Evelyn colorimeter with an uninoculated tube set at 100 as the blank; 10 ml. volume assay). Each assay tube contained 2.32 μg. of the liver concentrate (see text).

replace the factor required for the growth of *Leuc. citrovorum* depended also upon the presence of either xanthine, guanine, or hypoxanthine. Combinations of xanthine and guanine, or hypoxanthine and guanine were more active than any of the purines alone at the levels tested. The ability of thymidine (2 μg./tube) to replace the citrovorum factor apparently depends upon the presence of xanthine (or hypoxanthine), adenine, guanine, and uracil in the medium. The activity of thymidine was reduced considerably when tested in the presence of only one of the purines or derivatives or even in the presence of combinations such as guanine and xanthine or guanylic acid and xanthine. Crystalline vitamin B_{12}, when tested at a level up to 1 μg./10 ml. assay tube, was ineffective in replacing the purines required by *Leuc. citrovorum.*

Discussion

The purine and pyrimidine requirements of *Leuc. citrovorum* are different in certain respects from those reported for other microorganisms (6). Snell and Mitchell (6) reported that for the related organism, *Leuconostoc mesenteroides*, uracil was stimulatory and guanine appeared to be essential. In the presence of the essential growth factor, *Leuc. citrovorum* apparently does not require an exogenous source of pyrimidine but does of purine. This requirement of purine can be furnished by either xanthine or hypoxanthine and guanine, but not by adenine. It seems probable that the enzyme system present in many other microorganisms for the conversion of adenine to guanine, xanthine, or hypoxanthine is lacking in *Leuc. citrovorum.*

Adenine apparently cannot be converted to the other necessary purines but does have a sparing effect when the organism is cultured on suboptimal amounts of xanthine, hypoxanthine, or guanine. This indicates that perhaps part of the xanthine, hypoxanthine, or guanine is converted to adenine.

The purine requirement of *Leuc. citrovorum* is in contrast to the requirements of *Lactobacillus lactis* Dorner reported by Shive *et al.* (1) for which vitamin B_{12} apparently can replace both the thymidine and purines. For *Leuc. citrovorum,* vitamin B_{12} failed to replace either thymidine or the purines. Likewise, folic acid was ineffective in replacing the purines although Snell and Mitchell (6), Stokstad (7), and others (8,9) have shown that *Streptococcus faecalis* and *Lactobacillus casei,* in the presence of folic acid, do not require purine for growth, but that in the absence of folic acid, thymine and a purine are required.

Increased amounts of the concentrates of the citrovorum factor permitted some growth of the organism in the absence of purines, but at least a 10-fold increase was necessary to obtain a comparable growth. Although paper chromatographic analyses did not indicate any appreciable amounts of purines in the concentrates (4), it is conceiveable that they contributed sufficient amounts of the required purines to permit measurable growth of the organism.

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Summary

1. The purine and pyrimidine requirements of *Leuconostoc citrovorum* 8081 were investigated. The organism was found in the presence of the citrovorum factor to require for growth a source of either guanine, xanthine, or hypoxanthine. Any one of the three purines could replace or spare the other two, but xanthine appeared to be somewhat more active than guanine or hypoxanthine.

2. Adenine, uracil, and thymine were not required for growth of the organism. Adenine alone was inactive as a source of purine, but possessed some sparing effect when added in the presence of suboptimal amounts of xanthine, hypoxanthine, or guanine.

3. Guanosine and guanylic acid were generally less active than guanine for stimulating the growth of *Leuconostoc citrovorum*. Uric acid and other related purine compounds were inactive, while benzimidazole was somewhat inhibitory when the organism was grown on suboptimal amounts of hypoxanthine.

4. Vitamin B_{12}, folic acid, thymidine, or folic acid and thymidine could not replace the purine requirement of this organism.

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The Reaction of Proteins with Acetaldehyde

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Introduction

The reaction of proteins with formaldehyde has been largely elucidated in recent studies from this and other laboratories (1–4).[2] The reaction of proteins with reducing sugars, particularly glucose, has also been investigated intensively, because of its role in the non-enzymatic browning of many foods and other natural products (5). It seems almost certain that aldehyde-amine addition or condensation represents the first step in this type of browning. The nature of the aldehyde, however, is a determining factor, since it is almost impossible to provoke browning with formaldehyde. It was, therefore, surprising to find that acetaldehyde causes browning of protein solutions more rapidly and at lower temperatures than does glucose. Some observations concerning the mechanism of interaction of acetaldehyde and proteins will be presented here. The nature of the brown pigments built onto the protein remains to be investigated.

Materials and Methods

Crystalline bovine serum albumin (BSA) was a commercial preparation. The N-acetyl derivative was prepared with acetic anhydride in buffered solution as previously described (4). The guanidyl derivative (6) was kindly supplied by W. L. Hughes of the Department of Physical Chemistry, Harvard University Medical School; ovomucoid (7) by H. Lineweaver of this laboratory. The preparation of polyglutamic acid polyamide, and of the corresponding polyglutamyl poly(6-aminohexamethyleneamide) (abbreviated in Table I as "polyamide" and "polyamine," respectively) have been described (8,5).

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[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] For a review of the extensive literature, see (3) and (4).

TABLE I

Reaction of BSA Derivatives and Model Polypeptides with Acetaldehyde

Protein used	Reaction conditions[a] Concentration: Protein	Reaction conditions[a] Concentration: Acetaldehyde	Time	Comments	Analyses: N[b]	Analyses: Amino-N[b]
	per cent	*per cent*			*per cent*	*per cent*
BSA	8	12	2 hr.	Brown gel[c]	14.6	0.2
BSA	8	5	2 hr.	Brown gel[c]	15.5[d]	0.7
BSA	8	3.6	4 hr.	Brown gel, 4 hr.	14.7[e]	0.3
BSA	6	4.2	17 hr.	Brown gel, 8 hr.	14.0	0.3
BSA	6	3.15	17 hr.	Brown gel, 8–16 hr.	14.7	0.4
BSA	6	2.1	17 hr.	Brown gel, 8–16 hr.	15.0	0.5
BSA	6	1.05	17 hr.	Sol.[f]	15.5	0.8
BSA, control	—	—	—	—	16.1	1.2
Guanidyl BSA	6	3.5	4 days	Yellow sol.	17.2	—
Guanidyl BSA	5	4.2	9 days	Yellow sol.	17.5	—
Guanidyl BSA, control	—	—	—	—	18.3	0.2
Acetyl-BSA	5	4.2	9 days	Sl. yellow sol.	15.4	—
Acetyl-BSA, control		—	—		15.4	0.1
Polyamine[g]	8	3	5 days	Dark brown gel	13.9[e,i]	1.7
Polyamine[g]	8	1	5 days	Brown gel	16.0[e,i]	3.9
Polyamine[g], control	—	—	—	—	15.7	5.2
Polyamide[h]	7	4	5 days	Sl. yellow sol.	19.6	—
Polyamide[h], control	—	—	—	—	20.3	0.1

[a] Reactions were carried out at room temperature in solutions buffered with approximately 0.6 *M* phosphate, pH 7.6. The pH dropped to about pH 7 during the course of the reaction.

[b] All analyses are on moisture-free basis.

[c] The addition of 20% sucrose did not inhibit browning, gelation, nor the loss of amino-N under such conditions.

[d] 0.33% acetaldehyde could be recovered after acid hydrolysis.

[e] No acetaldehyde could be recovered after acid hydrolysis.

[f] Reaction mixture diluted to 1% protein gave 47% transmission (500 mμ), measured against a control solution treated similarly but containing no protein.

[g] Polyglutamyl poly-6-aminohexamethyleneamide (Compound I).

[h] Polyglutamic acid polyamide.

[i] These preparations contained 2-4% phosphorus. The analyses were calculated to the phosphoric acid-free basis.

The reaction conditions are indicated in the tables. The protein derivatives were isolated by thorough dialysis, followed by freeze-drying. Analyses were performed on the products after they had been equilibrated with laboratory air, and corrected for moisture. Nitrogen was determined by the Kjeldahl method (9), amino-N by the Van Slyke manometric method (10),[3] amide-N as previously described (1), and molecular weight by osmotic pressure (11). Acetaldehyde was determined according to Alexander (12). Guanidyl-groups were determined according to Brand and Kassell (13).[4]

Results

Acetaldehyde reacts rapidly with BSA at room temperature in solutions buffered at pH 7–8. The reaction is evident from the appearance of brown color and gelation of the reaction mixture. The isolated protein shows decreased solubility, lowered total nitrogen, and greatly lowered amino content (Table I). The latter finding indicates that the aldehyde has reacted with the amino groups of the protein. This conclusion is strongly supported by the finding that reaction does not occur if most of the amino groups of BSA have previously been blocked by acetylation.

Further evidence for the participation of the amino groups was sought by the use of model polypeptides of either very high or very low amino-N content. A polyamine prepared from polyglutamic methyl ester and hexamethylenediamine (I)

$$\begin{array}{l}\text{—CO—CH—NH—} \\ \qquad\quad | \\ \qquad (CH_2)_2 \\ \qquad\quad | \\ \qquad\ \text{CO—NH—}(CH_2)_6\text{—}NH_{2\,n}\end{array}$$

reacted with acetaldehyde, as indicated by browning of the reaction mixture and lowered total and amino-N contents in the isolated products. On the other hand, polyglutamic acid amide was isolated almost unchanged from the reaction mixture (Table I). Thus, amide groups and peptide bonds were unreactive under the conditions used.

Acetaldehyde seems to combine not only with the amino, but, to a certain extent, also with guanidyl groups. This was shown with a BSA preparation, the amino groups of which had been transformed to guanidyl groups. The extent of reaction, however, of the guanidylated BSA

[3] The possibility exists that acetaldehyde-browned products give some spurious amino-N, as has been shown to occur with glucose-browned products (5). Acetaldehyde, at the concentrations used, did not interfere with accurate amino-N determinations.

[4] These analyses were kindly performed by Dr. J. W. Pence of this laboratory.

was very much smaller than that of the original BSA, containing amino groups, and there was no appreciable browning (Table I). The potential reactivity of the guanidyl groups was also indicated by the observation that, at elevated temperatures, some browning occurred with protamine, which contains many guanidyl groups and no primary amino groups.

The reaction of BSA with acetaldehyde was found to be greatly dependent upon the concentration of the reactants, particularly of the acetaldehyde. This was evident from the rate of browning (Fig. 1) and also from the rate of loss of amino-N (Table I). In most instances the time necessary for the reaction mixture to set to a stiff gel was a further criterion to gauge the reaction rate. At 25°C., and concentrations of at least 4% of both reactants, gelation occurred within a few hours, but at 3°C. the brown reaction mixture (6% protein, 4.2% acetaldehyde) gelled only after 4 days. The protein, after isolation from gelled reaction mixtures, was insoluble in water, salt, or saturated urea solutions.

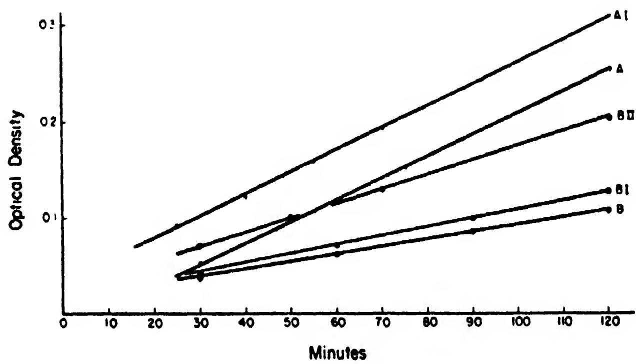

FIG. 1. A: 8% BSA, 4% acetaldehyde, final pH 7.3; AI: 8% BSA, 8% acetaldehyde, pH 7.0; B: 4% BSA, 4% acetaldehyde, pH 7.0; BI: 4% BSA, 8% acetaldehyde, pH 7.0; B II: 4% BSA, 12% acetaldehyde, pH 7.0.

In acid solution (pH 5 and 3.6), reaction, as indicated by browning, occurred very much more slowly than at neutrality (Fig. 2). The same intensity of color was obtained after 20 and 48, as compared to 2 hr. The rate constant of browning of BSA with 1.8 *M* acetaldehyde at pH 7.0 and room temperature was 2.3×10^{-3}, about 35 times as fast as that observed with glucose under comparable circumstances (5). The lack of an induction period under most of the conditions studied (Figs. 1 and 2) also differentiates the acetaldehyde from the glucose reaction.

The absorption spectrum of the protein browned with acetaldehyde showed a generalized adsorption, decreasing from high to very low values over the range of mμ = 350 to mμ = 600 (Fig. 3).

The gelation of BSA-acetaldehyde reaction mixtures is tentatively attributed to the formation of crosslinks between reactive groups of different protein molecules. Increased average molecular weights of glucose derivatives of BSA have been explained in similar manner (5), and the mechanism and the quantitative aspects of crosslinking through formaldehyde have been studied in detail (1,2,11). Thus, condensation was found to occur between amino methylol and a variety

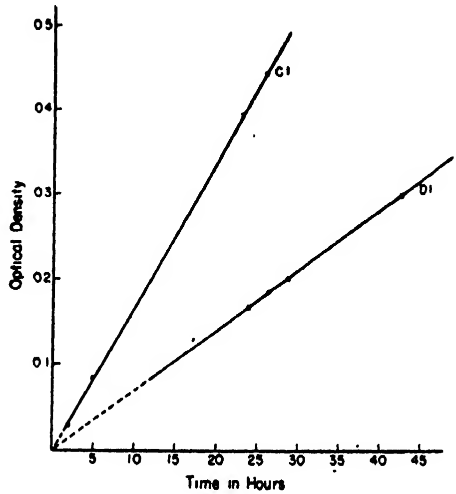

FIG. 2. Rate of browning of BSA (8%) with acetaldehyde (8%) at room temperature CI, pH 5.0; DI, pH 3.6.

of other protein groups of which the primary amide and guanidyl were both the most reactive and the most common. It was also found that crosslinking by formaldehyde between protein amino and amide or guanidyl groups is prevented in the presence of high concentrations of small molecular amides, guanidyl compounds, or amines (1). Similar experiments have now been attempted with acetaldehyde. The addition of methylguanidine sulfate to reaction mixtures containing BSA or ovomucoid led to the fixation of small amounts of this compound by the protein, as indicated by the Sakaguchi reaction (Table II). In contrast, no acetamide was bound under similar conditions, notwithstanding the

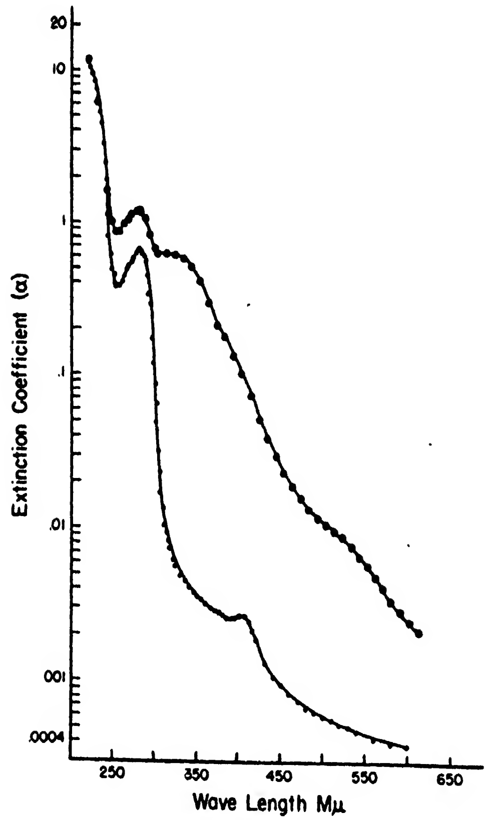

FIG. 3. Ultraviolet absorption spectra of BSA (lower curve) and the browned BSA-acetaldehyde derivative (upper curve).

fact that the gelation of BSA reaction mixtures was greatly delayed in the presence of this compound.

Direct proof for the ability of acetaldehyde to introduce crosslinks between protein molecules has come from determinations by osmotic pressure measurements of the average molecular weight of soluble derivatives (Table III). As in the studies with formaldehyde (11), BSA, and particularly ovomucoid, were found suitable proteins for this study; the latter remains water-soluble even during prolonged treatment with the aldehydes. The fact that acetamide did not prevent the increases in molecular weight produced with acetaldehyde (in contrast to its effect with formaldehyde) shows that crosslinking in this

TABLE II

Effect of Small-Molecular Compounds on the Reaction of Proteins with Acetaldehyde[a]

Protein[b]	Acetaldehyde conc.	Added compound[b]	Time	N[c]	Amino-N[c]	Added compound bound Equiv./10^4 g.[d]
	per cent			*per cent*	*per cent*	
BSA (8)	2	Methylguanid. sulf. (15)	6 hr.	15.4	0.7	0.5
BSA (8)	2	Acetamide (20)	5 hr.	15.5	0.7	0.0
BSA (8)	2	None	5 hr.	15.2	0.8	—
Ovomucoid (8)	4	Methylguanid. sulf. (15)	4 da.	12.6		0.5
Ovomucoid (8)	4	Acetamide (20)	4 da.	12.3	0.2	0.0
Ovomucoid (8)	4	None	4 da.	12.6	0.3	—

[a] Reaction conditions same as in Table I.

[b] Figures in parentheses represent concentrations (per cent).

[c] Untreated ovomucoid contains 13.9% N and 0.60% NH_2-N. For corresponding values for BSA see Table I.

[d] As determined by the Sakaguchi test for guanidyl groups (13), and amide-N analyses for acetamide introduced.

TABLE III

Apparent Molecular Weight of Acetaldehyde-Treated Proteins from Osmotic Pressure Measurements

Reaction conditions[a]	Apparent molecular weight[b]
8% BSA, 2% acetaldehyde, 2.5 hr.	116,000
8% BSA, 2% acetaldehyde, 20% acetamide, 3 hr.	138,000
Untreated BSA	70,000
8% ovomucoid, 4% acetaldehyde, 3 hr.	38,000
8% ovomucoid, 4% acetaldehyde, 4 days	175,000
	110,000 (6.7 *M* urea)
8% ovomucoid, 4% acetaldehyde, 15% methyl guanidine sulfate, 4 days	83,000 (6.7 *M* urea)
Untreated ovomucoid	29,000
	26,000 (6.7 *M* urea)

[a] Experiments performed at room temperature and pH 7.6–7.0 (see Table I).

[b] The solution used for the analyses contained 0.05 *M* pH 5.0 acetate and 0.05 *M* NaCl. When urea (6.7 *M*) was added, as indicated in parentheses, the buffer was pH 6.9 phosphate (0.05 *M*), final pH 7.7. The urea was used to exclude aggregation as a factor contributing to the increased molecular weight of protein derivatives (11).

case involves groups other than amide.[6] This was also indicated by the above finding that acetamide is not bound to the protein in the course of the reaction. On the other hand, the incorporation of methylguanidine suggested, and the partial inhibition of crosslinking between protein molecules by this compound supports the conclusion that guanidyl and amino groups are involved.

Some of the browned reaction products have been analyzed for reversibly bound acetaldehyde. As expected, and in conformity with similar results with glucose-browned BSA, none, or only a very small fraction, of the introduced aldehyde could be regenerated by acid hydrolysis (Table I). The fact that the aldehyde residue undergoes considerable further reactions after addition to amino groups is indicated by the finding that the change in the nitrogen content of the browned products corresponds usually to the addition of 3–4 acetaldehyde residues for each amino group blocked. In contrast, in glucose-browned BSA (5) there was an apparent 1:1 ratio of glucose residues bound and amino groups lost.

Another protein which was exposed to the action of various aldehydes was wool. The fact that, under our conditions, the only brown product was the protein was illustrated by the colorless appearance of the solutions in contact with the browned wool fibers. The same conclusion had been previously reached in regard to the browning of proteins with glucose (5). Acetaldehyde caused browning of wool within a few hours at room temperature, aldol only slightly less rapidly, but propionaldehyde much less rapidly. Acrolein, chloral, glucose, and benzaldehyde caused no browning at room temperature. Browning with acetaldehyde occurred in 50 and 96% ethanol, or in 50% acetone, but not in dry acetone. The mechanism of browning of a fibrous protein like wool appears to be similar to that of serum albumin; increasing acetylation of the wool with acetic anhydride rendered it progressively less "brownable."

A few model experiments were performed with alanine, acetaldehyde, acetamide, and methylguanidine sulfate (Table IV). They gave results in general agreement with those obtained with BSA: (1) the browning reaction was associated with a loss in amino-N; (2) it was dependent upon acetaldehyde concentration, pH, and temperature; (3) methyl-

[6] This was also indicated by the inability of acetaldehyde, in contrast to formaldehyde (1), to cause the gelation of a solution of gliadin in 50% acetic acid containing NH_4Cl.

guanidine, but not acetamide, appeared to enter into the reaction of alanine with acetaldehyde.[6]

In a few experiments, the relative rates of browning of some amino acids with acetaldehyde were compared. The results of one such experiment are summarized in Table V. α- and β-alanine browned rapidly,

TABLE IV

Reaction of Alanine[a] with Acetaldehyde

Reaction conditions: Acetaldehyde, Concentr.	Reaction conditions: Acetaldehyde, Millimole	pH	Time	Temp.	Residual Amino-N
per cent			*hr.*	°*C.*	*per cent*
9	1.5	7.3	2.5	40	62
9	1.5	5.0	2.5	40	90[b]
9	1.5	7.3	2	24	72[b,c]
3	0.5	7.3	24	40	91[b]
3	0.5	5.0	24	40	98

[a] 0.5 mM in 0.7 ml. of reaction mixture buffered with phosphate (pH 7.3) or acetate (pH 5). All reaction mixtures developed a yellow to dark brown color.

[b] Addition of acetamide (0.5 mM) to similar reaction mixtures had no effect on the apparent browning, nor on the extent of reaction (amino-N 88, 72, and 94% of original, respectively).

[c] Addition of methylguanidine (1.0 mM) as the sulfate decreased browning slightly; upon dilution (0.005 M in regard to the alanine), transmission was 44, as compared to 38%. The amino-N was 76% of the original.

serine and threonine much less readily, and α-amino isobutyric acid not at all. This is the same series of reactivities that had been found upon comparison of the abilities of amino acids to brown with glucose, or to fix formaldehyde irreversibly.[7] It seems probable that, in each case, reactions of the Mannich type involving the —CH(R)— group adja-

[6] It appeared of interest to determine whether acetaldehyde potassium disulfonate would react with amines in a manner similar to acetaldehyde. In experiments with BSA, as well as with alanine, no browning nor loss of amino-N was observed, even after prolonged reaction periods at 53°C. The sulfonated aldehyde was, however, always present only at low concentration, because of its low solubility in buffered aqueous solutions.

[7] Fraenkel-Conrat, H., Mohammad, A., and Olcott, H. S., unpublished experiments; see also French and Edsall (3).

TABLE V

Browning of Amines and Amino Acids with Acetaldehyde[a]

	Reaction time *hr.*	Extent of browning[b]
DL-Alanine[c]	2	100
β-Alanine	2	216
DL-Serine	2	39
DL-Threonine	2	26
Sarcosine	2	(10, yellow)
DL-Proline	2	(35, yellowish-brown)
DL-Hydroxyproline	2	(<35, yellowish-brown turbid)
α-Aminoisobutyric acid	6	0.8
DL-Serine O-sulfate	2	199
N-benzoyl-DL-serine	24	0
n-Butylamine	2	(<139, very turbid)
Ethanolamine	2	139
Diethanolamine	2	(<6, yellow, turbid)
Triethanolamine	6	(0.6, yellow)

[a] 0.5 m*M* of nitrogenous compound, neutralized in the case of the last 6 compounds by addition of 0.5 ml. of *N* acid or alkali, otherwise 0.5 ml. H_2O, 0.25 ml., pH 7.6, 3.4 *M* phosphate, and 0.5 ml. 15% (W/V) acetaldehyde (1.7 m*M*). Reactions allowed to proceed at 24 C. for time periods indicated. Reaction mixtures made to 10 ml. and, if necessary, rediluted (1:10).

[b] The reading of the brown color (on the Klett-Summerson photocolorimeter with a green filter) as compared to the alanine sample which is arbitrarily taken as 100% (actual reading of the "browned" 0.005 *M* alanine solution, 62).

[c] If the alanine was only 0.2 of the usual concentration but the acetaldehyde the same as usual, only 6% of the color was obtained (2 hr.). If both reactants were diluted 5-fold only 3% of the color was developed in 6 hr.

cent to the amino and carboxyl group are involved, and that a hydroxyl group on the next C-atom decreases the reactivity of the carbon chain (14). Sulfation of the hydroxyl group of serine, in turn, abolishes this effect. The carboxyl group appears not essential, but in its absence a hydroxyl group seems to favor the reaction. No typical brown but a yellowish color developed with secondary amines, and almost, and absolutely, no color with a tertiary and acylated amine, respectively.

Summary

Treatment of proteins with acetaldehyde at neutrality and room temperature causes browning within a few hours. With bovine serum albumin, the reaction mixtures gel. The rate of browning with acetal-

dehyde is about 35 times as fast as that observed with glucose under comparable conditions.

The reaction involves primarily the amino groups, and, to a much smaller extent, the guanidyl groups and causes some crosslinking, probably between these two types of groups, as shown by increases in the average molecular weight. Amide groups do not participate.

The reactions of amino acids or simple amines with acetaldehyde appear to be analogous to those observed with proteins.

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Studies on Cell Enzyme Systems. III. Effect of Temperature on the Constants in the Michaelis-Menten Relation for the Luciferin-Luciferase System [1]

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Introduction

In many reactions catalyzed by enzymes there is evidence for the formation of a compound or complex between the enzyme and the substrate before reaction can occur. Michaelis and Menten (10) have shown that if such a complex is formed the velocity, v, of the reaction will be given by

$$v = \frac{V\,(S)}{K_s + (S)}, \tag{1}$$

where (S) is the substrate concentration, V is the maximum velocity at high substrate concentrations, and K_s is the so-called Michaelis constant. This relation is found to give an accurate description of the dependence of the velocity of many enzymatically catalyzed reactions on the substrate concentration.

The effect of the temperature on the Michaelis constant might be expected to yield information on the mechanism of enzyme action. It has recently been shown (5) that the *in vitro* luminescent reaction of *Cypridina* luciferin and luciferasec an be analyzed in terms of the Michaelis-Menten theory. The data from this reaction are of sufficiently high precision to warrant a study of the effect of the temperature on the Michaelis constant. We have therefore investigated the relationship between the initial velocity of this luminescent reaction and

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substrate concentration as a function of the temperature. From the resulting Michaelis constants and maximum velocities we have calculated the thermodynamic constants related to the formation and decomposition of the enzyme–substrate complex.

Experimental

The general procedures used have been described in detail by Chase (5). In brief, the initial velocities of the reaction of luciferin catalyzed by luciferase were measured in terms of the amount of light produced per unit time. The light from the reaction vessel impinged upon a photoelectric cell and the total charge passed by this cell was allowed to accumulate in a condenser. The voltage resulting from this charge could then be measured at any desired time. The apparatus used was essentially the same as that described by Anderson (1).

The luciferase stock solution was obtained by extracting 5 g. of dry, powdered *Cypridina* organisms in 100 ml. of distilled water, filtering, and subjecting the filtrate to prolonged dialysis. While this enzyme preparation is by no means pure, all dialyzable material is presumably removed and a considerable amount of inactive protein is precipitated during the dialysis.

The luciferin stock solution was obtained by one cycle of purification, using Anderson's procedure (2).

Although measurements of the Michaelis constant over a wide temperature range are desirable, it is very difficult with this luminescent system to measure the reaction far from room temperature when the substrate concentration must be varied. At low temperatures and low luciferin concentrations the light emission is too small to be measured accurately, whereas at high temperatures the enzyme rapidly undergoes inactivation and, also, a considerable amount of the substrate disappears in a nonluminescent side reaction [Chase and Lorenz, (6)]. These factors so complicate the measurements that our experiments were necessarily restricted to 15° and 22°C. However, in order to obtain reliable values of the Michaelis constant from a study involving only two temperatures, four series of experiments were performed at each, with 5 to 7 individual luminescence runs making up each series. The procedure used for such a series of runs is described elsewhere (5).

Results

Table I gives the measured initial velocities of the luminescent reaction in mv./min. for the 55 individual runs. The values are grouped into four experimental series, the data in different series being obtained on different days.

The determination of V and K_s from the experimental data may be accomplished by the graphical method of Lineweaver and Burk (9), who have written Eq. 1 in the following form,

$$(S)/v = (S)/V + K_s/V \tag{2}$$

TABLE I

Initial Velocity of the Luminescent Reaction as a Function of Initial Luciferin Concentration

The latter is expressed as milliliters of luciferin stock solution present in 20 ml. of reaction mixture. Four sets of experiments are shown, each set consisting of a series at 15° and a series at 22°C.

Ml. of luciferin stock solution	Initial velocity in mv./min.							
	Exper. 125		Exper. 126		Exper. 127		Exper. 128	
	15°	22°	15°	22°	15°	22°	15°	22°
0.04	17	29	15	18	15	19	16	19
0.06	27	34	19	22				
0.08	26	38	23	33	26	36	24	31
0.10	33	43	26	37	27	38	26	35
0.20	43	72	44	62	43	58	40	60
0.40	59	102	56	91	58	87	55	82
0.80					73	122		106
0.90	73	131	76	123				
1.50					82	138	78	125

When $(S)/v$ is plotted against (S) a straight line should be obtained with slope $1/V$ and intercept K_s/V at (S) = zero. Fig. 1 shows such a plot of our data. The substrate concentration is in terms of milliliters of stock luciferin solution present in 20 ml. of reaction mixture. In order

TABLE II

Values of Maximum Velocity and of K_s *for the Eight Experimental Series*

These were computed from the slopes and intercepts of the lines which fit the data as plotted in Fig. 1. K_s is in terms of milliliters of luciferin stock solution present in 20 ml. of reaction mixture. For values in terms of molarity of luciferin see the text.

Exper. No.	22°C.		15°C.	
	Max. veloc.	K_s	Max. veloc.	K_s
	mv./min.		*mv./min.*	
125	167	0.26	84	0.17
126	169	0.34	93	0.24
127	168	0.34	96	0.24
128	147	0.30	90	0.23

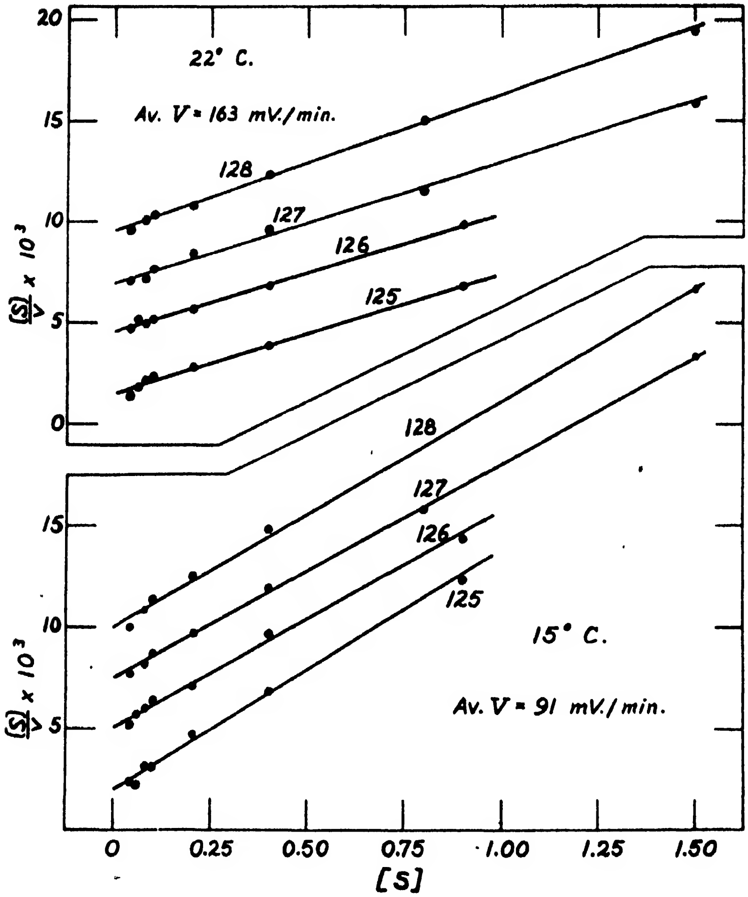

FIG. 1. The data of Table I plotted according to the equation,

$$(S)/v = (S)/V + K_s/V$$

(S) is expressed as milliliters of luciferin stock solution in 20 ml. of final reaction mixture, as actually used.

In order to avoid confusion in the graph, the origins of the ordinates for the series of experiments labelled "126," "127," and "128" have been shifted vertically upward on the (S)/v axis by 2.5, 5.0, and 7.5 units, respectively.

to show how each set of experiments fits the equation, the origin of the ordinate has been shifted vertically as explained in the legend.

Table II gives the values of the maximum velocity and of K_s computed from the slopes and intercepts of each of the lines which fit the

data as plotted in Fig. 1.[2] The average values of the maximum velocity at 22° and at 15°C. are 163 and 91 mv./min., respectively. 1.41 is the average of the individual ratios of K_s at 22° to K_s at 15°C. for each pair of experimental series.

Discussion

Significance of the Michaelis-Menten Equation

The Michaelis-Menten equation is usually interpreted according to the following reaction scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products}$$

where k_1, k_2, and k_3 are the specific rate constants of the reactions indicated. It was shown by Briggs and Haldane (3) that according to this scheme the constants in Eq. 1 are related to these k's as follows:

$$V = k_3 \tag{3}$$

$$K_s = (k_2 + k_3)/k_1 \tag{4}$$

If k_2, the rate of dissociation of the complex, is much greater than k_3, the rate of its decomposition into the reaction products, we have

$$K_s = k_2/k_1 \tag{4a}$$

The Michaelis constant can then be identified with the thermodynamic dissociation constant of the enzyme–substrate complex. The usual thermodynamic analysis can be applied to K_s and its derivative with respect to the temperature to obtain the heat and entropy of dissociation of the enzyme–substrate complex.

If, on the other hand, the complex decomposes more rapidly to give the reaction products than to give back the substrate (k_3 much greater than k_2), then

$$K_s = k_3/k_1 \tag{4b}$$

and the Michaelis constant is merely a ratio of rate constants. Even in this case it possesses a quasi-thermodynamic significance however, since, as Eyring (8) has shown, any specific rate constant can be expressed as

$$k = (RT/Nh)\,K^*$$

where K^* is a modified equilibrium constant between the reactants and the activated complex and R, T, N, and h are the Gas constant, the absolute temperature, Avoga-

[2] It can be seen from Table II that the two series of experiments labelled "125" give appreciably lower values of K_s than do the other series, all of which are consistent. We have no explanation for this and must attribute it to some at-present-unknown experimental factor operating on the day when these two sets of measurements were made. The ratio of K_s at 22° to K_s at 15°C. for these two series of experiments is, however, of about the same magnitude as the corresponding ratios from the other three pairs of series.

dro's number and Planck's constant, respectively. Thus, according to Eq. 4b, K_s is equal to the ratio of the equilibrium constants for the formation of the activated complexes in the two forward reactions of the Michaelis-Menten scheme.

$$K_s = \frac{K_3^*}{K_1^*} = e^{\frac{\Delta S_3^* - \Delta S_1^*}{R}} e^{\frac{-(\Delta H_3^* - \Delta H_1^*)}{RT}}$$

where K_1^*, K_3^*, ΔS_1^*, ΔS_3^*, ΔH_1^*, and ΔH_3^* are the equilibrium constants, entropies and heats of formation of the activated complexes in the two reactions. Application of the standard thermodynamic analysis to K_s will thus in this case give an apparent heat which is really the difference between the heats of activation of reactions 3 and 1. Similarly the apparent entropy obtained from K_s will really be the difference between the entropies of activation in the two reactions.

Analysis of the Constants in the Michaelis-Menten Equation for the Luciferin-Luciferase Reaction

In order to make a conplete thermodynamic analysis of the constants derived from the experimental data it is necessary to express these constants in standard concentration units. This requires that we find the molar concentrations of the enzyme and of the substrate in our solutions. Since neither the purity nor the molecular weight of the enzyme or substrate is accurately known, these concentrations can be only roughly estimated. Nevertheless, it will be found worth while to make the estimate.

The luciferin stock solution contained 2.4×10^{-5} g. of solids/ml. Luciferin very probably has a relatively low molecular weight; we shall take it to be 300 [Chase (4)]. If the stoek solution contained no impurity it would therefore be 8×10^{-5} M in luciferin. It is hardly likely that this figure is either too large or too small by more than a factor of three. Assuming this molarity, the Michaelis constant, K_s, is 0.88×10^{-6} moles/l. at 15°C. and 1.24×10^{-6} moles/l. at 22°C.

The reaction mixture contained 5×10^{-8} g. of protein/ml. Assuming that only half of this is enzyme [Chase, Schryver, and Stern (7)] and that there is one mole of prosthetic group on the enzyme per 20,000 g. of enzyme, we see that the concentration of enzymatically active groups in the reaction mixture is of the order of 10^{-9} M. This figure could well be in error by a factor of, say, five, but is more likely to be too large than too small.

It will also be necessary to make an estimate of the conversion factor from millivolts on the photoelectric integrator to moles of luciferin reacted. It is found that 0.03 ml. luciferin stock solution gives a total of 280 mv. at 22°C. This corresponds to 8.6×10^{-12}

moles of luciferin/mv. Chase and Lorenz (6) have found that about 10% more light is emitted at 15°C. than at 22°, so that at 15° we have 7.7×10^{-12} moles/mv.

Using these conversion factors we find for the maximum velocity in absolute units at 15° C., $k_3 = 0.58$ sec.$^{-1}$, and at 22° C., $k_3 = 1.17$ sec.$^{-1}$ These values of k_3 lead to an activation energy of 17,000 cal./mole and an entropy of activation of − 0.5 entropy units. Because of the uncertainty in the enzyme concentration, the entropy of activation may be too small by 3 entropy units, but this is smaller than the uncertainty in the entropy resulting from the experimental error in the temperature coefficient of k_3.

From the change in the Michaelis constant with the temperature we obtain an apparent heat of dissociation of the enzyme–substrate complex of 8,200 cal./mole and an apparent entropy increase on dissociation of 0.8 entropy units. If K_s is given by Eq. 4*a* rather than 4*b*, the latter figure is the entropy change when one mole of enzyme–substrate complex decomposes to give luciferin at a concentration of one mole/l. of solution. It is, however, desirable to eliminate from the entropy change that part which depends upon the entirely arbitrary choice of the units of concentration. This may be accomplished by subtracting the contribution of the partial molal entropy of mixing of the luciferin with the water in a one molar solution. This is given by $- R \ln x$, where x is the mole fraction of the luciferin at this concentration. Since in a one molar aqueous solution there are about 55 molecules of water for each molecule of solute, $x = 1/56$ and the entropy of mixing is 8.0 entropy units.

We shall call the entropy change corrected for the entropy of mixing in this way the *inherent entropy change* of the reaction. The inherent entropy of dissociation of the luciferin–luciferase complex is thus − 7.2 entropy units, provided that K_s is the true dissociation constant and not a ratio of the rate constants of two successive reactions. This would then be the entropy change when one mole of the complex decomposes to give pure enzyme and pure luciferin in the same state of hydration as in dilute aqueous solution.

An error of a factor of three in the molarity of the luciferin stock solution would result in an error of 2 entropy units in this entropy. The probable error from other sources is, however, larger than this, so that the assumptions regarding the purity and the molecular weight of the luciferin do not seriously affect these results.

SUMMARY

The rate of the *in vitro* luminescent reaction of *Cypridina* luciferin in the presence of luciferase has been studied as a function of the luciferin concentration at 15° and at 22° C. The Michaelis-Menten equation relating the rate and the substrate concentration is valid at both temperatures. From the effect of temperature on the constants in this equation thermodynamic constants relating to the formation and decomposition of the enzyme–substrate complex have been derived.

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The Amino Acid Composition of Gelatins, Collagens and Elastins from Different Sources [1,2]

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Introduction

The amino acid composition of gelatin and collagen has been extensively studied, but little attention has been given to possible differences in composition of these proteins from different sources.

Elastin prepared from cattle *ligamentum nuchae* has been analyzed in some detail by previous workers (1,2). However, a complete amino acid analysis has not been made nor has elastin from other species of animals or tissues been studied.

Collagen and elastin have unusual physical properties and chemical composition. These proteins are abundant and widespread throughout the higher animal organism and they are involved in many developmental and pathological changes.

The unique characteristics of the amino acid composition of collagen are its high content of glycine, proline, and hydroxyproline, coupled with the very low content of tyrosine, tryptophan, isoleucine, cystine, and histidine.

The unique characteristics of elastin are its high content of glycine, alanine, proline, and valine together with its low content of serine, tryptophan, the sulfur amino acids, the acidic amino acids, and the basic amino acids.

In the present paper are presented almost complete amino acid analyses of 4 gelatin, 12 collagen, and 6 elastin preparations.

[1] Taken in part from a dissertation presented to the Faculty of the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] This work was done during the tenure of a Life Insurance Medical Research Student Fellowship.

Materials

The source and preparation of the samples analyzed are listed in Table I.

TABLE I

Source and Preparation of Gelatins, Collagens, and Elastins

Source	Method of preparation
Gelatins	
1. Difco Bacto	————
2. Eastman purified calf-skin	————
3. Eastman purified pig-skin	————
4. Fish scale	Autoclaved fish scales (2 g./10 ml. water) 2 hr. at 15 lb. Evaporated filtrate to dryness.
Collagens	
1. Cattle hide[a]	$Ca(OH)_2$ and trypsin treatment according to a published procedure (3).
2. Cattle hide[a]	Extraction with cold 10% NaCl solution and acetone
3. Cattle bone[b]	Tissues obtained fresh. Separated from fat, flesh, and mucoids by mechanical means and by washing. Cut into small pieces and shredded in an ice water medium in a Waring Blendor. Suspended in 10% NaCl at 4°C. for 2 weeks with occasional change of solution. Washed. Suspended in $M/15$ Na_2HPO_4 at 4°C. for 3 days. Washed. Treated successively with acetone, ethanol, and ether each at 4°C. for 8–20 hr. Air-dried.
4. Cattle achilles tendon	
5. Pig achilles tendon	
6. Sheep achilles tendon	
7. Chicken tarsometatarsal tendon	
8. Cattle tail tendon	
9. Rat tail tendon	
10. Kangaroo tail tendon[c]	
11. Turtle (Cumberland terrapin, *Pseudemys elegans*) subcutaneous membrane	
12. Fishskin (Halibut)	

[a] We are indebted to Dr. R. M. Lollar and Dr. P. R. Buechler of Tanners' Research Council, University of Cincinnati, for two samples of cattle hide collagen.

[b] Before the usual treatment, fine shavings were obtained by drilling fresh bone under an ice-water mixture with a power drill. These shavings were suspended in 0.1 N HCl for four months with frequent changes of solution as in the procedure of Beek (4).

[c] Kangaroo tail tendon was kindly supplied us by Johnson and Johnson Company, New Brunswick, N. J., and by Dr. T. Salo, M. I. T.

TABLE I (*Continued*)

Source	Method of preparation
ELASTINS	
1. Cattle *ligamentum nuchae*	Prepared as were collagens, up to treatment with organic solvents. Elastins Nos. 1 and 2 subjected to 24 hr. in water at 100°C. with frequent changes of solution. Elastins Nos. 3–6 subjected to 40 hr. in 40% urea at 100 C. with frequent changes of solution. All elastins treated successively with boiling acetone, boiling ethanol, and warm ether. Air-dried.
2. Sheep *ligamentum nuchae*	
3. Cattle *ligamentum nuchae*	
4. Sheep aorta	
5. Cattle aorta	
6. Pig aorta	

The ash content of purified samples of the proteins was found to vary from 0.04–0.23% except in the case of cattle hide collagen which contained 0.5% ash.

EXPERIMENTAL

The microbiological assay with *Clostridium perfringens* BP6K was used according to the procedure of Boyd, Logan and Tytell (5) to determine leucine, isoleucine, valine, methionine, cystine, tryptophan, phenylalanine, tyrosine, threonine, glutamic acid, histidine, and arginine.

Microbiological assay with *Leuconostoc mesenteroides* P-60 was used for the determination of lysine, aspartic acid, glycine, and proline. Growth was determined turbidimetrically by a turbidity comparator (6).

Except for the method of determing growth the procedure of Henderson and Snell (7) was used in the assay of glycine. In the same manner the precedures of Dunn *et al.* (8), Henderson and Snell, and Sauberlich and Baumann (9) were used in the assay of lysine. Similarly the procedures of Henderson and Snell and Sauberlich and Baumann were used in the assay of aspartic acid and proline. The assay values obtained on representative samples under the different growth conditions were essentially the same.

Microbiological assay with *Leuconostoc citrovorum* 8081 was used according to the procedure of Sauberlich and Baumann (11) for the assay of alanine. The medium contained pyridoxamine, pyridoxal, and pyridoxine.

Serine was determined according to the procedure of Boyd and Logan (12). The values obtained probably include formaldehyde derived from hydroxylysine. For this reason the values presented for

serine were calculated after correction was made for an assumed 1.0% hydroxylysine content of all gelatins and collagens. No correction was made for elastin which is reported to contain no hydroxylysine (13). Collagen and gelatin are reported to contain 1.0–1.3% hydroxylysine (13,14).

Standards for assays were all Merck and Company products except *l*-proline which was from the Pfanstiehl Chemical Company. Recrytallized *dl*-alanine was used as standard for alanine determinations. All standards were stored in a desiccator over P_2O_5.

Protein hydrolyzates for microbiological assays were prepared as follows: About 0.5 g. of sample was dried overnight at 95°C. and weighed in glass-stoppered weighing bottles. Drying at 108°C. reduced the weight of representative samples of collagen less than an additional 1%. The protein was sealed in a 18 × 150 mm. pyrex test tube with 5.0 ml. 2 *N* HCl and autoclaved 10 hr. at 15 lb. The solution was neutralized, filtered, and brought to 50 ml. volume. For tryptophan assay, hydrolyzates were prepared according to the procedure of Stokes *et al.* (15).

For the determination of serine, the collagens and gelatins were refluxed 6 hr. with 6 *N* HCl (9 ml./50–100 mg. protein). Gelatin after 4 hr. refluxing yielded the same values as after 6 hr. Elastin refluxed under the same conditions yielded about 10% lower values after 18 hr. than after 6 hr.; because of these observations and those of Boyd and Logan, 6 hr. was chosen for the period of hydrolyzing elastin.

Nitrogen of samples dried overnight at 95°C. was determined after digestion of 50–100 mg. of a sample in 10 ml. H_2SO_4, 1.6 g. Na_2SO_4, 0.4 g. $CuSO_4 \cdot 5H_2O$, and 18 mg. $SeOCl_2$. Boiling of the digestion mixture was continued for six hours after clearing.

Results

As shown in Table II, there was little variation in the amino acid composition of most of the gelatins and collagens from various sources. However, one gelatin from a commercial source was significantly low in arginine and tyrosine.

Table IV contains some of the published "best" values for the amino acid composition of gelatin, collagen, and elastin. These values were chosen on the basis of their agreement with each other and the reliability of the method of determination. In certain cases the only values

TABLE II

The Amino Acid Composition of Gelatins, Collagens, and Elastins

	Nitrogen	Glycine	Alanine	Isoleucine	Leucine	Valine	Serine	Threonine	Proline	Phenylalanine	Tyrosine	Tryptophan	Methionine	Cystine	Histidine	Arginine	Lysine	Aspartic acid	Glutamic acid
	g./100 g. of protein dried at 95°C. for 16 hr.																		
GELATINS																			
Difco Bacto	17.6	25.7	8.6	1.5	3.1	2.8	3.2	2.0	16.3	2.3	0.91	—	0.92	0.09	0.85	8.3	5.2	6.4	11.5
Calfskin (Eastman)	17.4	26.9	8.7	1.9	3.1	2.6	2.9	2.2	14.0	1.9	0.14	—	0.85	0.05	0.63	6.4	5.2	6.9	12.1
Pigskin (Eastman)	18.0	30.5	9.2	1.5	3.2	2.7	2.9	2.2	16.3	2.1	0.69	—	0.80	0.09	0.67	8.8	5.1	6.3	11.7
Fish scale	17.6	27.4	—	1.2	2.5	2.2	3.5	3.3	14.1	2.2	0.72	—	2.2	0.17	1.01	8.9	4.9	6.5	11.4
COLLAGENS																			
Cattle hide (Prepn. No. 1)	17.8	27.5	8.8	2.2	3.2	2.7	2.8	2.2	15.8	2.3	0.89	—	0.89	—	0.62	8.7	5.3	7.0	11.4
Cattle hide (Prepn. No. 2)	18.1	29.3	10.0	2.1	3.2	2.7	2.8	2.2	16.5	2.4	1.00	—	0.94	—	0.74	8.6	5.5	7.5	10.8
Cattle bone	18.0	23.3	—	2.1	3.4	2.7	3.1	2.3	—	2.6	0.98	<0.01	0.72	0.11	0.64	9.0	5.5	—	11.2
Cattle achilles tendon	18.1	24.8	8.7	2.1	3.5	2.8	3.2	2.3	13.3	2.4	0.91	<0.01	0.84	0.11	0.75	9.0	4.9	6.7	11.9
Pig achilles tendon	18.1	26.6	8.7	1.7	3.4	2.7	3.1	2.3	14.3	2.4	0.96	<0.01	0.89	0.10	0.66	9.0	5.2	6.7	11.9
Sheep achilles tendon	17.9	25.1	9.1	1.7	3.4	3.0	3.0	2.5	14.1	2.3	0.89	<0.01	0.86	0.10	0.67	8.9	5.3	6.7	11.5
Chicken tendon	17.8	26.2	9.3	2.0	3.4	2.4	2.8	2.5	15.7	2.6	0.90	<0.01	1.16	0.12	0.68	8.9	5.5	6.7	12.2
Cattle tail tendon	17.8	28.0	8.7	1.9	3.6	3.2	3.2	2.3	14.9	2.4	0.98	—	0.89	—	0.80	8.7	5.3	6.9	11.7
Rat tail tendon	18.2	28.9	9.7	1.9	3.2	2.9	3.2	2.5	15.5	2.6	1.06	—	0.96	0.08	0.56	8.9	5.7	6.9	11.9
Kangaroo tail tendon	17.8	26.4	8.7	1.5	3.0	2.7	3.1	2.3	—	2.5	0.93	—	0.91	0.10	—	8.6	5.5	7.0	11.6
Turtle subcutaneous membrane	—	26.9	9.1	1.8	3.5	2.3	—	3.4	15.6	—	0.97	—	0.68	0.09	0.68	9.0	5.1	—	11.9
Fishskin	18.4	29.6	9.2	1.7	3.7	2.5	5.9	3.5	13.7	2.4	0.86	—	2.3	0.12	0.94	9.4	5.6	7.1	11.5
ELASTINS																			
Cattle *ligamentum nuchae*[a]	16.7	—	18.4	4.3	8.4	18.4	1.0	1.15	—	5.7	1.85	<0.01	0.03	0.25	0.05	1.05	0.40	—	2.2
Sheep *ligamentum nuchae*[a]	—	—	—	3.8	8.5	15.5	—	1.6	—	5.4	2.36	—	0.12	—	0.21	—	0.42	—	1.9
Cattle *ligamentum nuchae*[b]	17.1	29.9	18.9	4.0	8.7	17.4	0.82	0.96	17.0	5.0	1.61	<0.01	0.03	0.15	0.07	0.89	0.39	0.63	2.1
Sheep aorta[b]	—	27.9	20.0	3.6	8.1	18.4	—	1.16	15.8	5.0	2.10	—	0.0	—	—	0.90	0.37	0.61	—
Cattle aorta[b]	17.1	30.2	19.3	3.9	8.7	17.6	0.80	1.01	16.8	5.1	1.63	—	0.0	0.12	—	0.87	0.47	0.64	2.1
Pig aorta[b]	—	28.8	20.1	2.7	7.9	16.5	0.82	1.31	15.4	5.4	2.91	—	0.0	0.15	—	0.93	0.51	0.44	2.8

[a] Prepn. treated 24 hr. in water at 100°C.
[b] Prepn. treated 40 hr. in 40% urea at 100°C.

TABLE III

Standard Error and Number of Assays

	Nitrogen	Glycine	Alanine	Isoleucine	Leucine	Valine	Serine	Threonine	Proline	Phenylalanine	Tyrosine	Tryptophan	Methionine	Cystine	Histidine	Arginine	Lysine	Aspartic acid	Glutamic acid
	g./100 g. of protein dried at 95°C. for 16 hr.																		
Gelatins																			
Difco Bacto	0.18^{2}	1.0^{3}	0.30^{2}	0.07^{3}	0.10^{2}	0.13^{3}	0.01^{2}	—	0.92^{2}	0.05^{2}	—	—	—	—	—	—	0.22^{3}	0.21^{2}	—
Calfskin (Eastman)	0.05^{3}	—	—	0.05^{2}	0.20^{2}	0.10^{2}	0.05^{2}	0.01^{2}	0.30^{2}	—	0.045^{2}	—	0.045^{2}	—	—	0.05^{2}	0.21^{3}	0.06^{2}	0.20^{2}
Pigskin (Eastman)	0.11^{2}	0.05^{2}	—	0.15^{3}	0.15^{2}	0.20^{2}	0.03^{2}	0.05^{2}	—	—	0.025^{2}	—	0.016^{2}	—	—	0.20^{2}	0.24^{3}	0.08^{2}	0.15^{2}
Fish scale	0.04^{3}	1.4^{3}	—	0.05^{2}	0.05^{2}	0.10^{2}	0.05^{2}	0.10^{2}	0.40^{2}	0.09^{2}	0.010^{2}	—	0.010^{2}	—	—	0.30^{2}	0.15^{3}	0.39^{2}	0.15^{2}
Collagens																			
Cattle hide (Prepn. No. 1)	0.002^{3}	1.0^{3}	0.47^{2}	0.10^{3}	0.20^{2}	0.07^{3}	0.03^{2}	0.14^{3}	0.75^{3}	0.05^{2}	0.030^{2}	—	0.001^{3}	—	—	0.25^{4}	0.33^{3}	0.08^{2}	0.34^{4}
Cattle hide (Prepn. No. 2)	0.032^{3}	0.48^{4}	0.17^{3}	0.16^{4}	0.10^{2}	0.09^{4}	0.03^{2}	0.00^{2}	0.75^{3}	0.00^{2}	0.042^{2}	—	0.008^{3}	—	0.017^{3}	0.14^{4}	0.26^{3}	0.11^{3}	0.44^{4}
Cattle bone	0.050^{3}	—	—	0.14^{3}	0.10^{2}	0.00^{2}	0.04^{2}	0.06^{3}	—	0.40^{2}	0.071^{2}	—	0.020^{2}	0.010^{2}	—	0.00^{2}	—	—	0.06^{3}
Cattle tendon	0.17^{3}	1.4^{2}	0.18^{2}	0.09^{4}	0.10^{2}	0.05^{2}	0.05^{2}	0.00^{2}	0.48^{3}	0.09^{2}	0.10^{2}	—	0.018^{4}	—	0.030^{2}	0.09^{3}	0.29^{4}	0.01^{2}	0.64^{4}
Pig tendon	0.10^{3}	0.05^{2}	0.10^{2}	0.12^{3}	0.10^{2}	0.09^{2}	0.09^{2}	0.20^{2}	0.55^{4}	0.10^{3}	0.025^{3}	—	0.10^{4}	—	0.035^{3}	0.05^{3}	0.17^{4}	0.00^{2}	0.32^{4}
Sheep tendon	0.039^{3}	0.67^{2}	0.52^{3}	0.034^{3}	0.00^{2}	0.15^{4}	0.02^{2}	0.00^{2}	0.75^{3}	0.09^{3}	0.058^{3}	—	0.000^{2}	—	0.050^{2}	0.15^{2}	0.00^{2}	0.35^{2}	0.38^{4}
Chicken tendon	0.002^{3}	0.54^{3}	0.11^{2}	0.10^{3}	0.00^{2}	0.08^{4}	0.00^{2}	0.06^{3}	0.47^{3}	0.04^{3}	0.060^{2}	—	0.023^{4}	—	0.009^{3}	0.05^{4}	0.15^{2}	0.15^{2}	0.09^{3}
Cattle tail tendon	0.001^{2}	0.31^{3}	0.22^{3}	0.00^{2}	—	0.08^{3}	0.04^{2}	—	0.75^{3}	0.06^{3}	—	—	0.000^{2}	—	—	0.29^{3}	0.18^{4}	0.04^{3}	—
Rat tail tendon	0.12^{6}	0.43^{3}	0.45^{3}	0.52^{3}	0.10^{2}	0.05^{2}	0.05^{2}	0.05^{2}	0.65^{2}	0.05^{2}	0.025^{2}	—	0.027^{3}	0.000^{2}	0.015^{2}	0.23^{4}	—	—	0.32^{3}
Kangaroo tail tendon	0.023^{2}	—	0.65^{2}	0.05^{2}	—	—	0.05^{2}	0.06^{3}	—	—	0.035^{2}	—	0.027^{3}	0.007^{2}	—	0.32^{3}	—	0.05^{2}	0.45^{3}
Turtle subcutaneous membrane	—	1.6^{3}	—	0.05^{2}	—	—	—	0.10^{2}	0.92^{2}	—	—	—	0.025^{2}	0.000^{2}	—	0.15^{3}	0.14^{2}	0.08^{2}	0.20^{2}
Fishskin	0.16^{3}	0.87^{3}	0.25^{2}	0.10^{2}	0.10^{2}	0.20^{2}	0.07^{2}	0.03^{2}	0.05^{2}	0.05^{2}	—	—	0.000^{2}	—	0.030^{2}	—	0.21^{4}	0.11^{4}	0.05^{2}
Elastins																			
Cattle *ligamentum nuchae*[a]	0.053^{3}	—	—	0.27^{3}	—	1.1^{3}	0.01^{2}	0.05^{2}	—	0.15^{2}	0.050^{2}	—	0.010^{2}	0.015^{2}	0.010^{2}	0.05^{2}	—	—	0.20^{2}
Sheep *ligamentum nuchae*[a]	—	—	—	0.15^{2}	0.15^{2}	0.14^{2}	0.00^{2}	0.11^{2}	—	0.20^{3}	0.12^{2}	—	0.010^{2}	—	—	—	0.06^{2}	—	0.05^{3}
Cattle *ligamentum nuchae*[b]	0.13^{3}	0.9^{6}	0.12^{4}	0.07^{3}	0.09^{3}	0.07^{3}	—	0.10^{2}	0.85^{4}	0.11^{2}	0.039^{3}	—	—	—	0.020^{2}	—	0.03^{4}	—	0.09^{3}
Sheep aorta[b]	—	0.9^{3}	0.00^{2}	0.14^{3}	—	—	—	—	0.87^{3}	0.05^{2}	—	—	—	—	—	—	—	—	—
Cattle aorta[b]	0.001^{2}	1.0^{6}	0.69^{3}	0.15^{2}	0.26^{2}	0.20^{2}	0.01^{2}	0.05^{2}	0.75^{4}	0.00^{2}	—	—	—	—	—	0.06^{2}	0.04^{2}	0.005^{2}	0.11^{2}
Pig aorta[b]	—	1.3^{4}	0.52^{3}	0.15^{2}	0.30^{2}	0.78^{2}	0.00^{2}	—	0.98^{3}	0.05^{2}	0.10^{2}	—	—	—	—	0.03^{2}	0.02^{2}	0.020^{2}	0.02^{3}

The superscripts refer to the number of analyses made.

The values in this table are the standard errors, $S\bar{x}$. $S\bar{x} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{N(N-1)}}$, where $\Sigma(x-\bar{x})^2$ is the summation of squares of the individual deviations from the mean and N is the number of analyses. No values are entered in the table when no analysis, or a single analysis, was made.

[a] Preparations treated 24 hr. in water at 100°C.

[b] Preparations treated 40 hr. in 40% urea at 100°C.

TABLE IV

Published "Best" Values for the Amino Acid Composition of Gelatins, Collagens, and Elastins

	Gelatins	Collagens	Elastins
		g./100 g. protein	
Glycine	26.0–27.0 (17)[a]	26.2–27.2 (17,18)	29.4 (1)
Alanine	8.7–9.6 (11,19,20,21)	9.5 (20)	0–6.0 (1,22)
Isoleucine	1.4–1.7 (15,23,25)	——	3.4 (2)
Leucine	3.2–3.6 (15,23,24,25,26, 27,28)	3.5 (26)	7.3–8.6 (2,28)
Valine	2.5–2.7 (15,23,25,29)	3.4 (20)	13.5–13.8 (1,2)
Serine	3.2–3.8 (20,30,31)	3.2–3.7 (20,31)	——
Threonine	1.9–2.2 (15,20,23,31)	2.3–2.4 (20–31)	1.1 (2)
Proline	14.8–17.6 (17,20,32,33)	15.1–17.5 (17,20,33)	15.2–15.6 (1,2)
Phenylalanine	2.2–2.6 (15,20,23,34,35)	4.2 (20)	3.34–4.8 (2,36)
Tyrosine	0.49–1.1 (21,23)	1.0 (37)	1.4–1.6 (1,2)
Tryptophan	0.0–0.003 (24,38)	0.0 (39)	0.0 (1)
Methionine	0.6–1.0 (24,40,41)	0.8 (39)	0.3–0.38 (1,2)
Cystine	0.1–0.2 (24,42)	——	0.23–0.6 (1,2)
Histidine	0.6–1.0 (15,19,20,23,24, 34,43)	0.6–0.9 (18,20,43)	0.0–0.04 (1,2
Arginine	8.6–9.3 (20,29,43,44)	8.8 (20,43)	1.0–1.1 (1,2)
Lysine	4.1–5.9 (19,20,23,24,34, 43,45)	4.1–5.0 (18,20,43,46)	0–0.5 (1,2)
Aspartic acid	5.5–6.8 (30,47)	6.3 (39)	0.0–0.6 (1,2)
Glutamic acid	10.2–11.7 (23,47,48,49)	11.3 (39)	2.7–3.3 (2,22)

[a] The numbers in parentheses are literature references.

available were entered. It will be noted that the values in Table II were, in general, in agreement with those from the literature. Valine and alanine contents of elastin were found to be distinctly different from those previously reported. Fishskin collagen contained 50–100% more methionine, serine, and threonine than mammalian or avian collagen. Fish scale gelatin was similar to fishskin collagen in methionine and threonine content. Turtle subcutaneous membrane collagen was similar to fishskin collagen in threonine content. It has been reported (16) that the methionine, serine, and threonine content of isinglass from fish swim bladders is greater than that of gelatin.

Elastins from different species of animals and tissues varied little in amino acid composition; pig aorta elastin, however, differed in isoleucine, glutamic acid, and tyrosine content. It is possible that these

differences are due to impurities. If there is a contaminating protein, it must be outstandingly low in isoleucine.

Table III lists the standard errors for the assay values of Table II. The total amino acids as (*a*) g./100 g. protein, (*b*) total amino acid residues, and (*c*) per cent of total nitrogen accounted for are shown in Table V.

TABLE V

Total Dry Weight and Total Nitrogen of Preparations

	Total amino acids	Total of amino acid residues	Total nitrogen accounted for
	g./100 g. protein	%	%
Gelatins			
Difco Bacto	115.9	97.5	99.1
Calfskin	112.5	94.6	99.2
Pigskin	120.4	101.3	102.1
Fish scale	116.8	98.8	101.2
Collagens			
Cattle hide (Prepn. No. 1)	117.9	99.9	100.6
Cattle hide (Prepn. No. 2)	121.8	102.9	102.4
Cattle bone	113.9	96.6	94.9
Cattle achilles tendon	113.8	96.2	95.2
Pig achilles tendon	116.3	98.4	96.8
Sheep achilles tendon	114.8	97.2	96.3
Chicken tendon	118.7	99.6	100.8
Cattle tail tendon	119.2	100.7	100.9
Rat tail tendon	122.2	103.1	101.4
Kangaroo tail tendon	116.0	98.2	99.0
Turtle subcutaneous membrane	119.1	99.5	—
Fishskin	125.6	106.0	104.0
Elastins			
Cattle *ligamentum nuchae*[a]	111.4	92.4	94.7
Sheep *ligamentum nuchae*[a]	108.9	90.8	—
Cattle *ligamentum nuchae*[b]	110.5	92.2	92.5
Sheep aorta[b]	109.0	90.6	—
Cattle aorta[b]	111.2	92.4	92.5
Pig aorta[b]	108.5	90.1	—

To obtain these figures the data in Table II were used together with the best published values for hydroxyproline and hydroxylysine. Also included was the average amino acid content where the value was missing in Table II.

[a] Preparations treated 24 hr. in water at 100 C.

[b] Preparations treated 40 hr. in 40% urea at 100 C.

Criteria of purity of fibrous proteins are undeveloped and it may be recognized that similarities or differences in preparations may arise fortuitously as a result of an admixture of varying proportions of proteins or other material. Similar preparative methods applied to different tissues do not always assure preparations of similar purity. However, it is noteworthy that relatively simple procedures have yielded materials from such divergent sources as the turtle, the kangaroo, and the cow, which exhibit the characteristic amino acid pattern of collagen. The same is true of elastin from different sources.

Summary

1. An almost complete amino acid analysis was made of 4 gelatin, 12 collagen, and 6 elastin preparations.

2. Gelatins may show variable composition and be unreliable for studying collagen composition.

3. Collagens from fish and turtle differed from those of higher organisms in content of one or more of the three amino acids: methionine, serine, and threonine.

4. Collagens from different tissues and from different mammalian sources and an avian source were similar in amino acid composition.

5. Elastins from different species of animals and tissues were similar in amino acid composition.

6. A preparation of pig elastin, possibly because of impurity, was relatively low in isoleucine and high in tyrosine and glutamic acid.

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The Effect of Soybean Growth Inhibitors on the Availability of Methionine for Growth and Lipotropism [1]

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Introduction

The presence of trypsin inhibitors in raw soybeans (4,5,8,14) and the deleterious effect of concentrates of the antitryptic factors on the growth of experimental animals (3,9,13) have been adequately demonstrated, and inactivation of these inhibitors by proper heat treatment has been associated with improvement in the nutritive value of soybean protein (23). It has been suggested (18) that the inhibitor exerts its growth-depressing effect by retarding the enzymic release of methionine in the gastrointestinal tract, thus making this amino acid unavailable for simultaneous utilization with the other essential amino acids. Evidence has been recently presented (6,12,22), however, which indicates that an inhibition of proteolytic hydrolysis cannot be the major causative mechanism. The present report gives the results of an investigation designed to determine more about the nature of this effect, especially in relation to the well-known effectiveness of methionine as a supplement to unheated soybean meal (1,10). The extent to which the trypsin inhibitor interferes with the availability or utilization of methionine as measured by growth (weanling rats) and by the lipotropic action of methionine in low-choline diets has been determined.

Experimental

The approach to the present investigation was based on a recent study by Treadwell (20) wherein a differentiation between the growth and lipotropic requirements

[1] This paper reports research undertaken by the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number *253* in the series of papers approved for publication. The views and conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of the Army.

for methionine in the absence of choline is reported. For optimum growth Treadwell found 1000 mg. of methionine/100 g. of diet to be necessary when the protein level was 18.6%, whereas the total methionine requirement for growth and lipotropism at that protein level was found to be between 1300 and 1500 mg./100 g. diet. For the present study, modification of Treadwell's diet was necessitated in order to incorporate sufficient raw soybean meal to demonstrate growth inhibition, and, at the same time, to minimize the soybean meal's contribution of choline to the basal diet. As a compromise between those two considerations, raw[2] or autoclaved[3] soybean meal (approximately 50% protein) at a level of 10%, and vitamin-free casein at a level of 7.5% were provided as the sources of dietary protein. For the present experiment, a protein level of 12.5% was used because it was believed that this would give the most sensitive measure of protein efficiency (2). In addition to protein, the basal diet contained, in per cent: lard, 40; salt, U.S.P. 2, 5.0; cystine, 0.1; and, as mg.-%: thiamine, 0.25; riboflavin, 0.50; niacin, 2.0; pyridoxine, 0.25; calcium pantothenate, 2.0; inositol, 10.0; *p*-aminobenzoic acid, 5.0; biotin, 0.01; folic acid, 0.10; 2-methyl-1,4-naphthoquinone, 0.10; and sufficient sucrose to make up to 97.5%. Adequate amounts of vitamins A, D, and E were administered twice weekly as a corn oil mixture of α-tocopherol and Haliver oil.

TABLE I

Growth and Lipotropism as Affected by Methionine in the Presence of Soybean Growth Inhibitors

Total methionine in diet[a] (mg./100 g.)	Gain in weight[b] (g./g. protein consumed)		Liver lipides[b] (g./100 g. dry wt.)	
	Raw soybean	Autoclaved soybean	Raw soybean	Autoclaved soybean
600	2.79±.04	3.76±.12	39.2±3.6	41.2±4.8
1000	2.64±.21	3.47±.17	19.9±1.3	20.4±1.3
1500	2.84±.14	3.43±.14	16.2±1.1	19.6±1.4
1500[c]		2.68±.16		14.3±1.0
2500	1.74±.18	2.54±.13	12.9±0.7	11.8±0.6

[a] Includes "effective" methionine content of basal, unsupplemented diet (see text).
[b] Average of each group (7 animals/group) ± the standard error of the mean.
[c] Plus 1.8% trypsin inhibitor preparation.

Calculation of the amount of supplemental methionine to be added was based on the methionine and choline values of the basal diet, which were found to be 257 and 31 mg./100 g., respectively, by the methods of Lyman, *et al.* (17), and of Engel (7). Using Treadwell's data (20), it was estimated that the choline requirement of 200 mg. could be met by about 600 mg. of methionine; hence, the total "effective" methionine content of the basal diet was considered to approximate 350 mg./100 g. The calculated quantities of DL-methionine necessary to provide final levels of 600, 1000, 1500, and 2500 mg./100 g. of the raw or autoclaved soybean diets were adjusted to 2.5 g. with

[2] Nutrisoy XXX, Archer-Daniels-Midland Co., Minneapolis, Minnesota.
[3] Autoclaved for 20 min. at 15 lb. pressure.

sucrose and added to 97.5 g. of the basal diet. An additional diet composed of autoclaved soybean meal plus 1500 mg. methionine/100 g. diet was further supplemented with 1.8% trypsin inhibitor concentrate prepared according to the procedure of Klose *et al.* (13) without an equivalent reduction in protein or sucrose. There were, therefore, nine diets in all (see Table I).

Nine groups of weanling male rats (Sprague-Dawley), consisting of 7 animals per group and having initial weights of 42–48 g., were fed the diets described above *ad lib.* for a period of 18 days. All animals were individually caged so that individual records of food consumption could be obtained. At the end of the experimental period, the animals were anesthetized, and the livers excised and weighed on previously tared aluminum foil dishes. After thorough maceration, the moist tissues were dried to constant weight under vacuum at 70°C. The dishes containing the dried tissues were inserted directly into fat extraction thimbles, and extracted overnight with petroleum ether. After careful evaporation of the solvent on a steam bath, the receiving flasks were dried to constant weight.

Diets containing the autoclaved soybean meal consistently supported better growth[4] than the unheated meal even in the presence of excessive amounts of methionine (2500 mg.-%) to the point where growth was adversely affected. The addition of the trypsin inhibitor concentrate to the diet containing the heated meal also produced the expected inhibition of growth. Methionine in excess of 600 mg.-% produced no further improvement in growth of rats receiving raw or autoclaved soybean meal.

Liver lipides, however, decreased progressively as the level of methionine was increased. The most marked lipotropic effect occurred when the methionine was increased from 600–1000 mg.-%. The lipotropic effect of 1500 mg.-% methionine was not significantly greater than that of 1000, although increasing the methionine to 2500 mg.-% produced a more significant decrease in liver lipides. The consistent failure to obtain significant differences in liver lipides between diets containing raw and autoclaved soybean meal at any level of methionine was of special interest. Adding the trypsin inhibitor to the autoclaved soybean meal diet containing 1500 mg.-% to methionine not only failed to increase the liver lipides above that of a similar diet without added inhibitor, but actually reduced the lipide content to some extent.

Discussion

Comparison of the results obtained using raw and autoclaved soybean meal diets with respect to growth and lipotropism shows that

[4] Apparent differences were analyzed for significance by the *t* test (18), and only those *t* values with a *P* value of less than 0.01 were considered significant.

heating caused a substantial improvement in growth of protein efficiency without a concomitant effect on liver lipides. Similarly, the added trypsin inhibitor depressed the growth of rats receiving autoclaved soybean meal without affecting the availability of methionine for lipotropism. It may be concluded, therefore, that the growth-depressing effect of the trypsin inhibitor is not related to an interference with the amount of methionine absorbed from the gastrointestinal tract, a finding which is in complete agreement with the observation that the actual absorption of sulfur (11) or methionine (18) from the intestinal tract of rats fed raw or autoclaved soybean is the same.

Although groups of animals receiving the basal, unsupplemented diets were not included in this study, sufficient evidence is available from studies reported elsewhere (1,10) that the nutritive value of such diets is improved by supplementation with methionine. Thus, the following typical results (16) were obtained when diets containing 12.5% protein derived from raw or autoclaved soybean meal were supplemented with methionine. Inasmuch as Treadwell (20) has re-

Diet	Protein efficiency
Raw soybean meal	1.33
Raw soybean meal+0.6% methionine	2.42
Autoclaved soybean meal	2.62
Autoclaved soybean meal+0.6% methionine	3.01

ported that methionine up to 1000 mg.-% produced a progressive increase in growth, the failure of methionine in excess of 600 mg.-% to effect further improvement in growth was somewhat unexpected. This difference in the growth-promoting effect of supplemental methionine is probably related to the higher level of protein in Treadwell's diet (18.6%) compared with that in this study (12.5%). As subsequently pointed out by Treadwell (21), the effectiveness of supplemental methionine may be limited by the availability of other amino acids when the protein intake is at a lower level.

The addition of the trypsin inhibitor concentrate to the autoclaved soybean diet caused a marked decrease in growth even when methionine was present in amounts far in excess of the level adequate to satisfy the need for methionine for growth at the level of protein fed. In an analogous manner, excessive amounts of methionine when added to diets containing raw soybean meal did not restore their nutritive value to the corresponding level of methionine-supplemented diets containing

autoclaved soybean meal. This finding may be interpreted as verification of the results of *in vitro* digestion studies previously reported showing that a decreased rate of release of methionine cannot be the only factor involved (15) and that the soybean trypsin inhibitor exerts an effect through a mechanism unrelated to the availability or utilization of methionine. The improvement in the nutritive value of raw soybean effected by supplemental methione may be, to a certain extent, a reflection of the fact that even properly heated soy protein is charaterized by a deficiency of methionine (1). The mode of action of the trypsin inhibitor in relation to the effectiveness of methionine supplementation of raw soybean meal will be the subject of a future detailed report.

Summary and Conclusions

1. The presence of methionine at effective levels of 600, 1000, 1500, and 2500 mg.-% in a low-choline, 12.5% protein diet containing raw or autoclaved soybean meal caused a progressive decrease in liver lipides of weanling rats.

2. The levels of liver lipides were equal for the same level of methionine intake regardless of whether the soybean protein was raw or autoclaved.

3. Differences in protein efficiency were not parallel to the changes in liver lipides described above. The growth-promoting value of diets containing raw soybean meal was consistently less than similar diets containing autoclaved soybean meal. This difference persisted in spite of the presence of more than adequate amounts of methionine necessary for growth.

4. Addition of trypsin inhibitor concentrate to the autoclaved soybean diet containing 1500 mg.-% methionine caused a definite decrease in rate of growth which was not accompanied by any significant change in liver lipides.

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The Thiobarbituric Acid Reagent as a Test for the Oxidation of Unsaturated Fatty Acids by Various Agents [1]

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Introduction

Aerobic incubation of a number of animal tissues produces a compound which on heating with thiobarbituric acid yields an orange-red product (1). If ascorbic acid is added to the incubating tissues more of the product is formed (2); and conversely, certain tissues of scorbutic guinea pigs produce smaller amounts of the colored product than corresponding tissues of normal animals when incubated without added ascorbic acid (3). Linolenic acid both in the free form and in the phospholipide molecule gives the same color reaction as tissues (4) and also forms an increased amount of the colored compound on incubation with ascorbic acid. In view of the fact that the oxidation of several lipide compounds can be catalyzed by ascorbic acid, and by mercapto compounds and ultraviolet light as well (5–11), it was quite possible that lipides other than linolenic acid would give the colored compound and that agents other than ascorbic acid might catalyze the reaction. These possibilities have been examined. Since thiobarbituric acid may combine with ketoses (12) and aldehydes (13,14) the specificity of the reaction has also been studied by testing sugars and straight-chain aldehydes.

The thiobarbituric acid test has been found in the present study to be especially sensitive to changes produced in certain unsaturated fatty acids by ultraviolet light. This suggested that fatty acids in skin exposed to ultraviolet irradiation might also be altered. Experiments reported here indicate that this is the case in human and mouse skin.

[1] Aided by a grant from the Williams-Waterman Fund.

Experimental

The following fatty acids were tested: pure methyl linolenate and methyl arachidonate (Hormel Foundation); linoleic acid (Eimer and Amend); oleic and stearic acids (Merck). They were dissolved in 95% ethyl alcohol and 0.2 ml. of this solution was added to every 2.0 ml. of phosphate buffer at pH 6.0 to give a concentration of $2.7\text{–}3.0 \times 10^{-4}$ M. Ascorbic acid when used was added in buffer so that the final concentration was 0.18 mg./ml. The emulsions were shaken in flasks at 37° in air. With incubation for periods of 12 hr. or longer, the emulsions were not entirely stable, especially in the more acid solutions.

At intervals, 2.0 ml. aliquots were removed; 1.0 ml. of 20% trichloroacetic acid and 2.0 ml. of 0.67% thiobarbituric acid solution were added and the mixture heated for 10 min. in a boiling water bath. The tubes were then cooled, centrifuged, and the color was read in a Fisher electrophotometer with filter 525-B. Incubated controls were used to correct for absorption due to any faint cloudiness of the solution. The density of the color produced with the thiobarbituric acid reagent follows Beer's Law at relatively low concentrations only. Since the densities shown in the graphs are plotted without correction for the deviation, the higher values and the differences between curves at the higher values are minimized. The recorded densities are taken from a logarithmic scale on the electrophotometer.

A Hanovia ultraviolet lamp without filter operating at 110 volts and 5 amp. was used for the irradiation of fatty acid emulsions, mouse skin, and human sweat. Solutions containing $1.35\text{–}1.5 \times 10^{-4}$ M fatty acid were shaken in large open test tubes at 37.5° at a distance of 24.0 cm. from the quartz tube source to the surface of the solution. The depth of solution did not exceed 10 mm. Tubes containing control samples were covered completely with aluminum foil and incubated simultaneously. Pieces of skin scraped free of fat, were pinned out on paraffin blocks, placed in a finger bowl and just covered with Ringer-Locke solution. The bowl was placed in a constant temperature bath at 37.5°; and the distance from the light source to the skin was 16.5 cm.

The effects of incubation of methyl linolenate and linoleic acid in the presence and absence of ascorbic acid are shown in Fig. 1. Incubation of methyl linolenate without ascorbic acid produced a compound which gave a positive test with thiobarbituric acid and its rate of production was greatly increased by the presence of ascorbic acid. Incubation of linoleic acid alone produced a negligible amount of the chromogen. With ascorbic acid there was an increase, though very much less than with linolenate. It is possible that this result may in part be due to contamination of the linoleic acid with traces of linolenic acid. Incubation of oleic acid, methyl arachidonate, stearic, and palmitic acids with and without ascorbic acid produced no chromogen. A crude preparation of C_{20} unsaturated fatty acids with a fish-like odor showed a marked increase in chromogen on incubation and an increase in the rate of formation in the presence of ascorbic acid. The amount was such that it cannot be attributed to contamination with linolenic acid.

Mercapto compounds are known to catalyze the oxygen uptake of linoleic and linolenic acids and phospholipides (5–8). Cysteine and glutathione also markedly accelerated chromogen formation from linolenate (Fig. 2). The effect on linoleic acid was much less striking, and no effect was produced on oleic acid and arachidonate. The catalytic effect is much greater at pH 3.4 than at pH 6.0; and this has also been

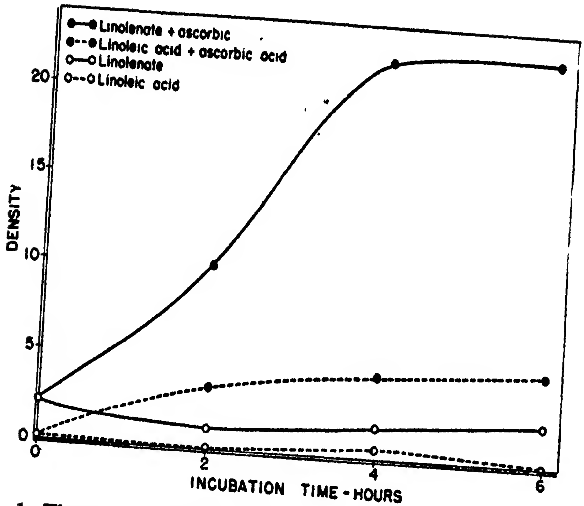

FIG. 1. The effect of ascorbic acid on chromogen production from methyl linolenate and linoleic acid at pH 6.0.

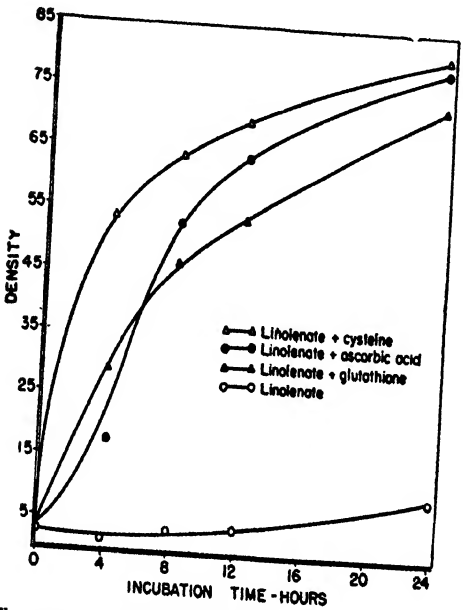

FIG. 2. The effect of cysteine, glutathione, and ascorbic acid on chromogen production from methyl linolenate at pH 3.4.

observed in the earlier work on oxygen consumption (5,6). Controls showed that cysteine and glutathione did not affect the development of the color by the reagents.

It is well known that exposure of unsaturated fatty acids to ultraviolet light catalyzes their autoxidation. The effect of ultraviolet light on chromogen formation has been studied on emulsions of saturated and unsaturated fatty acids irradiated for various periods. The results for several of these are shown in Fig. 3. Ultraviolet light greatly accelerated the production of the chromogen from methyl linolenate and also from methyl arachidonate, though to a smaller degree. It has little effect on linoleic, oleic, palmitic, and stearic acids.

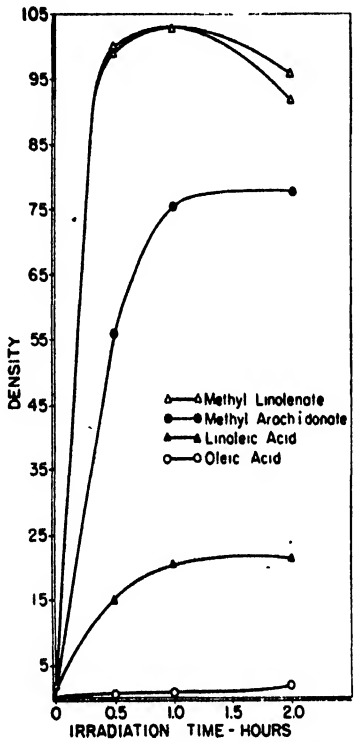

FIG. 3. The effect of ultraviolet irradiation on chromogen production from methyl linolenate, methyl arachidonate, linoleic acid, and oleic acid at pH 6.0.

The increased effectiveness of ultraviolet light over ascorbic acid in altering the fatty acid molecule is shown by the fact that ascorbic acid was without appreciable effect on arachidonate and also by the greater degree of change produced in linolenate by ultraviolet light. After ultraviolet irradiation the thiobarbituric acid test will detect changes in 0.2 μg. of linolenate/ml. at pH 6.0, whereas the maximum sensitivity after 24 hr. incubation with ascorbic acid is approximately 16 times this amount.

Plaisance (12) found that certain sugars after heating with HCl react with thiobarbituric acid. The reaction has been studied under the conditions used for tissues by

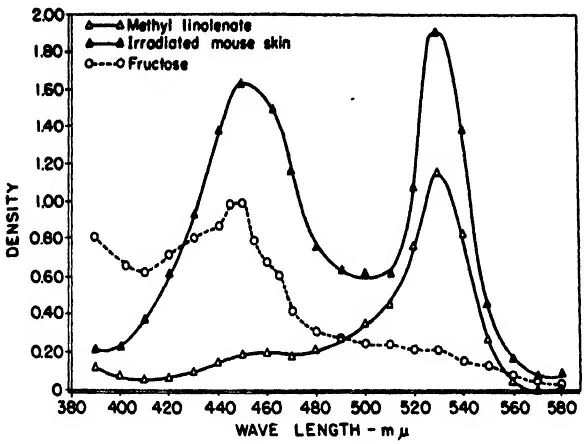

FIG. 4. The absorption spectra of the colors produced with thiobarbituric acid by methyl linolenate oxidized by ascorbic acid or ultraviolet light, by mouse skin irradiated with ultraviolet light, and by fructose.

incubating sugars in a final concentration of 0.067 *M* in buffer at pH 6.0 for 3 hr. at 37.5°. The color with thiobarbituric acid is yellow except in the case of arabinose which gives a pale pink. The absorption spectrum shows a peak at about 450 mμ for fructose (Fig. 4) and 520 mμ for arabinose. On standing a precipitate is formed, as pointed out by Plaisance. The relative color density as measured prior to precipitation is given in Table I. Of the single sugars, fructose produced the most color. Sucrose gave somewhat less than the sum of the colors of its constituent sugars. Ascorbic acid increased the amount of chromogens slightly in all except dextrose. In addition to the difference in hue the sensitivity of the test for sugars is of a different order of magnitude from linolenate. Accordingly, the sugars would ordinarily not seriously interfere with tests for fatty acids.

TABLE I

Color Production by Various Sugars with Thiobarbituric Acid

Incubation for 3 hr. at 37.5° in Na-K phosphate buffer pH 6.0. Sugar concentration 0.067 *M*; ascorbic acid concentration 0.94 *M*. Color density was measured with a Fisher electrophotometer with filter 425-B. All colors were yellow except D-arabinose which was pale pink.

Sugar	Na-K phosphate buffer	Na-K phosphate buffer + ascorbic acid
Galactose	22.8	28.7
D-Arabinose	16.8	22.2
Maltose	7.5	10.9
Dextrose	8.8	8.4
Fructose	57.8	62.3
Sucrose	53.7	58.6

Qualitative tests were carried out on short-chain aldehydes and the reactions are indicated in Table II. Glyoxylic acid is of interest in that a red color similar to that given by linolenic acid is produced. However, the absorption spectrum shows two maxima at 525 and 550 mμ whereas linolenate has a single peak at 530 mμ.

TABLE II

Reactions of Aldehyde with Thiobarbituric Acid

0.05 ml. of each aldehyde was heated with 5.0 ml. of reagents as indicated in the text. The third column gives the results on heating with trichloroacetic acid in the absence of thiobarbituric acid (TBA).

Aldehyde	With TBA	Without TBA
Formaldehyde	Soln. colorless	Soln. colorless
	Ppt. white	Ppt. none
Glyoxylic acid	Soln. bright pink	Soln. colorless
	Ppt. none	Ppt. none
Acetaldehyde	Soln. light yellow-brown	Soln. colorless
	Ppt. brown	Ppt. none
Aldol	Soln. light brown	Soln. faint yellow
	Ppt. dark brown	Ppt. none
Crotonaldehyde	Soln. light yellow	Soln. faint yellow
	Ppt. orange-brown	brown globule formed

The fact that ultraviolet light catalyzes the production of the chromogen in linolenate and arachidonate made it of interest to determine whether a similar reaction would occur when skin is exposed to ultraviolet radiation. The skin of white mice was shaved and two equal pieces cut. One piece was exposed to ultraviolet radiation for half an hour and the temperature controlled so that it remained the same as that of the unexposed piece. At the end of the exposure both pieces were immersed in the thiobarbituric acid reagent and allowed to stand at room temperature for 24 hr. or longer. In every experiment a deeper color developed in the exposed skin, indicating that ultraviolet light catalyzed the reaction. The difference could also be demonstrated by measuring the color density of the reagents in which the skin was immersed. The absorption spectrum of the color obtained from the skin had a maximum which corresponded to that of linolenate (Fig. 4). A strong yellow component was also present. Extraction of the skin with a boiling alcohol–ether mixture (15) before the addition of thiobarbituric acid prevented the formation of the characteristic color. The yellow component however remains after the extraction. It has an absorption maximum at 450 mμ (Fig. 4) and is, therefore, not produced by a fatty acid.

Human skin also contains substances which react with thiobarbituric acid and are altered by ultraviolet light. This was shown in the following manner. Sweat was collected directly in test tubes from the arm and trunk region of three male subjects after thoroughly cleaning the skin. A 2.5 ml. sample was tested with the thiobarbituric acid reagent and found to give a small color value. Exposure of the sweat to ultraviolet light for 30 or 60 min. at 37.5° gave values approximately 2–5 times greater than those in the corresponding unexposed solutions. The color values for nonirradiated samples varied from time to time in the same individual, as might be expected. The absorption spectrum had the same maximum as that for mouse skin.

Discussion

The autoxidation of unsaturated fatty acids and its catalysis by ultraviolet light probably involves the formation of peroxides and ketones (9,11). The course of the oxidation can be followed by measuring the oxygen consumption, and by this means it has been shown that ascorbic acid and mercapto compounds have a catalytic action (5–8). Chromogen production by fatty acids, particularly linolenic, as indicated by the thiobarbituric acid test parallels these previous studies in that this is also an oxidative reaction (1,2) and is catalyzed by ascorbic acid, mercapto compounds and ultraviolet irradiation.

The chemical reaction underlying the thiobarbituric acid test has not been completely elucidated. Since peroxides are formed under the conditions of our experiments these may be involved; and we have found that di-*tert*-butyl peroxide does give a color with the same absorption maximum as linolenate. (The statement previously made (4) that no color was obtained with this peroxide is incorrect.) Certain aldehydes also react with thiobarbituric acid to give different colors depending upon the compound. These include the sugars and some short-chain aldehydes. Thiobarbituric acid will also unite with tissue aldehydes as shown by the acid fuchsin test (14) and aromatic aldehydes (13). The blocking action of semicarbazide and phenylhydrazine on the thiobarbituric acid reaction (1,2) also points to aldehydes or ketones.

The sentivity of the thiobarbituric acid test for oxidized fatty acids, particularly linolenic, deserves emphasis. Under physiological conditions and in the absence of ultraviolet light, the thiobarbituric acid test may be considered primarily as an index of the amount of oxidized linolenic acid present and perhaps certain still unidentified C_{20} acids. Small amounts of linoleic acid would not contribute significantly to the values. Again in the case of the catalytic action of mercapto compounds, linolenate was outstanding in its sensitivity, with linoleic acid showing a slight response, and with no change with oleic acid, and arachidonate, palmitic, and stearic acids. The sugars are sufficiently different in their color reactions and sensitivity so as not to interfere with the tests. It is therefore possible to say that if a positive test is obtained with thiobarbituric acid on tissues or in the presence of ascorbic acid or mercapto compounds that it is probably due primarily to an oxidation product of an unsaturated fatty acid; and of the saturated and unsaturated fatty acids of 16 and 18 carbon atoms, linolenic acid is most sensitive. A

high sensitivity may also be found for certain of the C_{20} unsaturated acids since a crude preparation of these acids also gave high color values, though this was not true of arachidonate.

Kohn and Liversedge (1) showed that thiobarbituric acid reacts with sulfadiazine and 2-aminopyrimidine to give a color with the same absorption characteristics as that given by linolenic acid. Since these compounds are not normally present in the body, they would not interfere with the specificity of the test as applied to normal tissues.

Summary

1. Methyl linolenate after autoxidation or oxidation catalyzed by ascorbic acid, mercapto compounds or ultraviolet light yields a compound which reacts with thiobarbituric acid to produce a characteristic color. At pH 6.0 the intensity of the color is about 16 times greater after ultraviolet irradiation than after catalysis by ascorbic acid or mercapto compounds, and amounts of the order of 0.2 μg. of methyl linolenate per ml. can be detected after ultraviolet irradiation.

2. Under the same conditions linoleic acid produces very much less color and this may be due to the presence of small amounts of linolenic acid.

3. Methyl arachidonate produces no color after incubation with ascorbic acid or mercapto compounds but does so after exposure to ultraviolet light. Oleic and stearic acids are inactive, but a crude mixture of C_{20} acids gives a color after incubation with ascorbic acid.

4. Certain aliphatic aldehydes and sugars react with thiobarbituric acid to give colors. The color given by glyoxylic acid approximates most closely that given by linolenic acid. The absorption spectrum of the former has two peaks, at 525 and 550 mμ, that of the latter has one peak at 530 mμ.

5. Mouse skin and human sweat give the color characteristic of linolenic acid. After irradiation by ultraviolet light the color values of both are increased.

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The Acceleration of the Thermal Denaturation of Tobacco Mosaic Virus by Urethan at Normal and Increased Pressure [1]

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Introduction

On the basis of considerable kinetic evidence it appears that an important physiological action of urethan on enzyme systems is to cause both reversible and irreversible denaturations of the protein (1,2,3). Because of the importance of certain urethans medicinally and particularly in the treatment of some types of cancer (4,5) it has seemed desirable to obtain more information about the action of urethans on typical protein systems. Direct evidence has now been obtained that urethan catalyzes the thermal denaturation of tobacco mosaic virus, a pure protein whose thermal denaturation under normal (6,7) and increased pressure (7) has already been studied. The data indicate that several—possibly four—reactions are involved, and it is suggested that urethan molecules either catalyze or accelerate the normal reactions with hydroxyl or in some way actually replace one or more of the hydroxyls.

A hydrostatic pressure of 7000 lb./in.^{2} is qualitatively somewhat less effective in retarding the total rate of denaturation with urethan than the normal thermal denaturation.

Methods

The technique used in the previous work on tobacco mosaic virus (TMV) denaturation involved determination of uncoagulated virus at various stages of the denatura-

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tion process by means of direct Kjeldahl nitrogen analysis (7). In the presence of urethans, however, this technique is not readily applicable because of the high nitrogen content of the urethans themselves. Correction for this nitrogen, moreover, was unsatisfactory because the Kjeldahl method used did not digest the urethan completely nor consistently. The virus samples 6.0 mg./ml. in 0.1 *M* phosphate buffer of pH = 7.00, were denatured in stainless steel bombs at 68.8°C. as described in a previous article (7) except that the rubber dams were replaced with tiny rubber stoppers to ensure the exclusion of oil. The entire contents of each bomb were then pipetted and washed into a tared centrifuge tube and centrifuged for about 5 min. at 5000 r.p.m. The supernatant fluid was decanted carefully with a special pipet (a medicine dropper with a long drawn-out tip) into a second tared centrifuge tube which was then immersed in boiling water for 5 min. to complete the coagulation of the protein. Meanwhile the original precipitate was washed with distilled water by thorough stirring. A drop of dilute wetting agent[4] was added to the second tube. Both tubes were then recentrifuged. The supernatant from the second precipitate was discarded, but the supernatant from the washing of the original precipitate was used to wash the second precipitate. This washing was also held in boiling water for 5 min. to precipitate any soluble protein obtained from the washing of the first precipitate. The washed second precipitate was recentrifuged after addition of a drop of dilute wetting agent and the supernatant discarded. The two precipitates were dried in an oven at about 60° and the fraction undenatured obtained by the ratio of the weight of the second precipitate to the combined weights. This procedure automatically compensated for differences in the charges of the individual bombs and for any slight leakage during pressure experiments. Consistent urethan data even with the above procedure proved very difficult to obtain largely because sporadic traces of oil or tiny metal fragments would remain in the centrifuged, washed samples.

Results

The range of concentrations of urethan (ethyl carbamate) investigated extended from 0.0125–0.55 *M*, the former causing a barely measurable acceleration at normal pressure and the latter making the reaction so fast that higher concentrations of the drug could not be studied with any quantitative accuracy. The results of a typical experiment with one concentration of urethan at normal and at 7000 lb./in.^{2} are plotted in Fig. 1, showing that within the limits of normal experimental error the rate is first order at either normal or increased pres-

[4] The precipitates obtained by heating the supernatant from the first precipitate were very fluffy and considerable errors occurred at first because of material which formed a film on the surface and did not settle during centrifugation. This difficulty was overcome by adding a drop of 1:10 dilution of the wetting agent, "Ultra Wash" after boiling. If a drop of undiluted wetting agent were added before boiling, *no* precipitate formed whatsoever. This phenomenon is perhaps related to the increased thermal stability, and increased resistance to urea denaturation, of bovine serum albumin in the presence of fatty acids and certain other compounds (8,9).

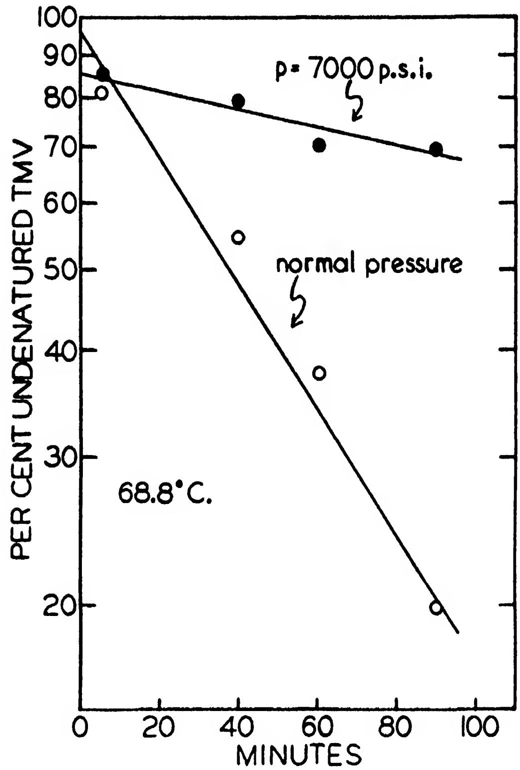

Fig. 1. Denaturation of tobacco mosaic virus at 68.8°C. in 0.1 M phosphate buffer 0.125 M in urethan. Log_{10} per cent undenatured virus against time in minutes. Two simultaneous experiments are shown, one at atmospheric pressure, the other at 7000 lb./in.^{2} as indicated. Best straight lines fitted by eye.

sure as already established for thermal denaturation alone (7). Similarly obtained first order rate constants are plotted against molar concentration of the drug in Fig. 2, on a log–log scale. The points represent the values obtained from the experiments while the smooth curves were calculated on the basis of the hypothesis described below.

Interpretation

The closeness of the pressure curve to a straight line of slope 3 suggested that at 7000 lb. pressure, over the concentration range studied, the reaction is predominently one involving three molecules of urethan. The atmospheric curve is asymptotic at the upper end to a

line also of slope 3 and at the lower end to the horizontal, *i.e.*, the normal thermal denaturation rate in the absence of urethan. The atmospheric curve could not be fitted with these two parameters, but if the reasonable assumption is made that in the concentration range between these extremes other reactions involving one and two molecules of urethan are involved, the rate may be expressed as follows:

$$-d[\mathrm{TMV}]/dt = k[\mathrm{TMV}],$$

where

$$k = k_0 + k_1(U) + k_2(U)^2 + k_3(U)^3.$$

Of these parameters k_0 is known from independent direct experimental determination and k_3 is the slope of the line to which the curve is asymptotic at its upper end; k_1 and k_2 are disposable and were chosen to fit the data.

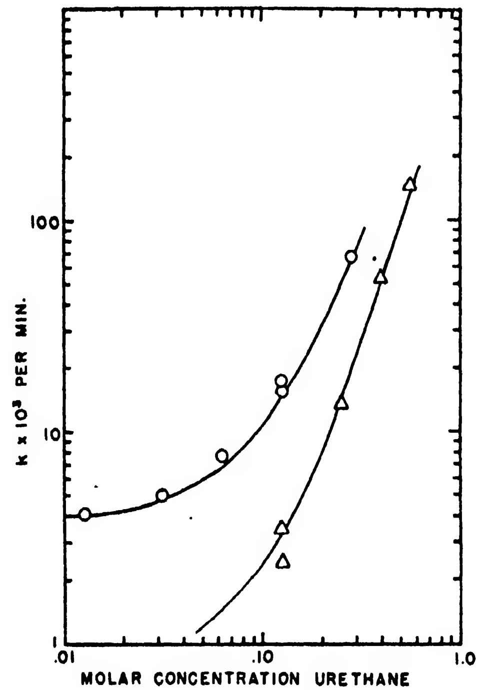

FIG. 2. Denaturation at 68.8°C. of TMV in 0.1 M phosphate buffer with varying amounts of urethan at atmospheric and 7000 lb. pressure. $\log_{10}$ reaction rate constant (multiplied by 1000) against $\log_{10}$ molar concentration of urethan. The circles represent points at atmospheric pressure, the triangles at 7000 lb./in.[2] Theoretical curves fitted as described in the text.

The rate at 7000 lb. is representable by an identical expression, with different constants:

$$k' = k'_0 + k'_1(U) + k'_2(U)^2 + k'_3(U)^3.$$

Again k'_0 is known from direct determination and k'_3 is the upper asymptote of the pressure curve. From the thermodynamic relationship

$$d \ln k_i/dP = -\Delta V_i/RT,$$

one can determine $\Delta V\ddagger$ of the reaction and use it to calculate k' from k (*cf.* 7). In the normal reaction $\Delta V\ddagger$ is 100 ml. (7). The $\Delta V\ddagger$ of the k_3 reaction can be calculated from k_3 and k'_3 and is approximately 50 ml. If $\Delta V\ddagger$ values are assumed for the k_1 and k_2 reactions the corresponding k' values can be calculated. It seems reasonable from previous results (*cf.* 7) and from the k_0 and $k_3\Delta V\ddagger$'s to assume values between 50 and 100 ml. For the present approximation, however, 100 ml. was assumed, the k'_1 and k'_2 values calculated and the pressure curve of Fig. 1 drawn.

The good fit of the two curves is, of course, not surprising in view of the large number of parameters. Of these the k_0, k'_0, k_3 and k'_3 values may be considered as accurate within the experimental limitations, while the k_1 and k_2 values are only approximations and their existence is justified only to the extent that they are kinetically plausible and required to fit the atmospheric data. The fact that normal thermal denaturation requires three hydroxyls (10) and the involvement here of three urethans—which may admittedly be a coincidence or an experimental limitation—suggests the following mechanism of competitive reactions (with the alternative interpretations I and II).

$$\mathrm{TMV} + 3(\mathrm{OH})^- \xrightarrow{k_0} \mathrm{TMV\ denatured}$$

$$\mathrm{TMV} + a(\mathrm{OH})^- + \mathrm{U} \xrightarrow{k_1} \mathrm{TMV\ denatured}$$

$$\mathrm{TMV} + b(\mathrm{OH})^- + 2\mathrm{U} \xrightarrow{k_2} \mathrm{TMV\ denatured}$$

$$\mathrm{TMV} + c(\mathrm{OH})^- + 3\mathrm{U} \xrightarrow{k_3} \mathrm{TMV\ denatured}$$

$$\mathrm{I}\ a = b = c = 3, \qquad \mathrm{II}\ a = 2, b = 1, c = 0.$$

Decision between these coefficients could be made on the basis of a study of the effect of pH on the denaturation with urethan. Although several points of critical interest are raised by these alternatives the present data are insufficient to justify further discussion. Each of these

steps should be interpreted in terms of the more detailed discussion of the k_0 reaction in the report on normal thermal denaturation (10). The constants, determined from previous work and graphically, as described above, are as follows:

	k_0	k_1	k_2	k_3
Atmospheric pressure	3.79×10^{-3}	2.2×10^{-2}	2.9×10^{-1}	1.9
7000 lb. pressure	0.87×10^{-3}	0.38×10^{-2}	0.53×10^{-1}	0.68
Approximate $\Delta V\ddagger$	+100 ml.	+100 ml.	+100 ml.	+50 ml.

Acknowledgment

The authors wish to thank Dr. Jacinto Steinhardt for helpful suggestions.

Summary

The thermal denaturation of tobacco virus at 68.8°C., pH = 7.00, is accelerated by urethan at both normal and increased hydrostatic pressure. Analysis of the data indicates that in the presence of urethan the total rate of denaturation probably depends upon four independent reactions: (*a*), the thermal denaturation itself, proceeding with a volume increase of activation of about 100 ml./mol; (*b*) and (*c*) urethan-catalyzed reactions, involving a similar or possibly somewhat smaller volume change and the participation of one or two urethan molecules; and (*d*) a urethan-catalyzed reaction proceeding with a smaller volume change of activation and involving the participation of three molecules of urethan. The extent to which each of these reactions predominates depends upon the concentration of the drug and the hydrostatic pressure; at low concentrations (ca. 0.125 *M*) the denaturation with urethan is strongly retarded by 7000 lb./in.^{2}, while at high concentrations (ca. 0.55 *M*), the rate, which is much faster, is much less affected by this pressure.

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Kinetics of the Action of the Sodium Salt of 2,4-Dichlorophenoxyacetic Acid on the Germ Lipase of Wheat [1,2]

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Introduction

The importance of the use of the 2,4-D group (2,4-dichlorophenoxyacetic acid, its sodium salt and other derivatives) as selective herbicides is shown by a production total of 8,000,000 lb. during 1947 (1). Considerable information has been obtained on specificity of the herbicide on plant families from field experiments: however, practically no information is available on the chemical action of this compound on the normal reactions of the plant. This research was undertaken in an effort to provide information on the subject of why the 2,4-D group generally kills broad leaf plants yet does not kill the narrow leaf species. Hagen (2) and coworkers have found that the sodium salt of 2,4-D does inhibit the action of ricinus lipase upon olive oil. Therefore, a study of the effect of the sodium salt of 2,4-D upon wheat germ lipase was undertaken to provide a basis for comparison of the effect of 2,4-D upon a lipase of a narrow leaf and of a broad leaf species of plants.

Experimental

Materials

A homogeneous wheat germ meal for use throughout this study was prepared from fresh wheat germ obtained from the North Dakota State Mill and Elevator. A 500 g.

[1] The data in this paper are taken from a thesis presented by Orville J. Kvamme to the Faculty of the Graduate School of the North Dakota Agricultural College in partial fulfilment of the degree of Master of Science.

[2] Published by the permission of the Director of the North Dakota Agricultural Experimental Station.

sample of fresh wheat germ was defatted for 3 days in a large Soxhlet extractor. The germ was dried and ground in a Wiley mill to pass a 40-mesh screen. The ground germ was then sifted through a 50-mesh screen, which removed most of the bran. The germ was dry-ground in a ball mill for 24 hr. The powdered meal was again defatted in the Soxhlet extractor for 24 hr. and dried. The resulting product was stored in the refrigerator. Water extractions of this wheat germ meal were made by stirring 1 g. of meal in 25 ml. of water at 0°C. for 15 min. The mixture was then placed in a centrifuge for 15 min. at 3000 r.p.m. The supernatant liquid was decanted and kept in a frozen state until required for use. The enzyme in the frozen state is stable over long periods of time (3).

The substrate used (glycol monoacetate) was synthesized by esterifying glycol with acetic anhydride in the presence of pyridine. The product, which was purified by a fractional distillation, had a boiling point of 183°C. (uncor.). An acetyl group determination indicated a 40% experimental value, in comparison to a 41% theoretical value.

The sodium salt of 2,4-D was made by treating the free acid obtained from the Baker Chemical Co. with an excess of NaOH and purified by recrystallization in water. The salt obtained was the monohydrate.

Method

All experimental work was carried out in the conventional Warburg constant volume respirometer. The experimental technique, as routinely used in the course of this study was as follows: The enzyme extract was placed in the main compartment of the Warburg vessel, followed by $NaHCO_3$ solution and other additions. The substrate, dissolved in bicarbonate solution of the same molarity as that of the solution in the main compartment, was pipetted into the side arm of the vessel. The reaction mixture was 0.025 *M* with respect to the $NaHCO_3$, and was kept in equilibrium with a gaseous mixture of 5% CO_2 and 95% N_2 by volume which resulted in a pH of 7.4 for the reaction mixture. This gaseous mixture was attained by using an evacuation procedure (4). After gassing for 3 min. with this mixture of CO_2 and N_2, followed by temperature equilibration for 10 min., the contents of the side arm were tipped in and the evolution of CO_2 during the first 50 min. was noted at 5 min. intervals. Between the readings, the system was shaken at 100 oscillations/min., which promotes a rapid gas exchange between the fluid and the gaseous phase. To correct for changes in atmospheric pressure and the temperature of the water bath, a thermobarometer was used. When an inhibitor was used in the reaction mixture, enzyme-inhibitor contact was made 15 min. before beginning the run.

By the use of the proper flask constant, the indicated pressure change of the system was converted into μl. of gas evolved at 0°C. and 760 mm. pressure. The rate of CO_2 evolution was used as the measurement of reaction velocity. It may be noted that, if it is desired to obtain the velocity constants in moles of substrate hydrolyzed per unit time, the following equation may be used:

$$k = \frac{v_{CO_2}}{V_{CO_2}} \cdot \frac{1}{V_{\text{solution}}\, t},$$

k = velocity constant in moles/l.-min.

In the above equation, v_{CO_2} is the volume of CO_2 evolved in time t from a solution sample of volume V. V_{CO_2} is the molal volume of the gas at the experimental temperature and pressure.

It was determined that evolution of a suitable amount of CO_2 for the range of the manometer resulted from a reaction mixture consisting of 1 ml. of enzyme extract, 1.5 ml. of 0.0375 M $NaHCO_3$ and 0.5 ml. of 1.8 M glycol monoacetate solution which was 0.0375 M with respect to $NaHCO_3$. It was necessary to shake the flasks during the course of the reaction, since otherwise the volume of gas evolved was not a linear function of reaction time. Under these conditions the reaction was of zero order during the time observed. The rate of reaction was proportional to the concentration of the enzyme.

Results

It was suspected that there would be some autohydrolysis (*i.e.*, hydrolysis which proceeds by a non-enzymic mechansim) of the glycol monaocetate in the presence of the $NaHCO_3$ buffer. For apparently identical conditions the rate constants for autohydrolysis differed markedly, whereas those for enzymic hydrolysis coincided within experimental error. Typical examples of this situation are given in Table I. A study of autohydrolysis at varied substrate concentrations was also made. Three trials were made at each concentration and readings were taken at 5 min. intervals. The rate of hydrolysis which best fits all of the data was determined by statistical methods (5). The different values which were calculated in this manner, are shown in Table II. Consideration of this table shows that the rate of autohydrolysis is not a linear function of substrate concentration.

The energy of activation of the wheat germ lipase in its action upon glycol monoacetate was 8 kg.-cal./mole for the temperature range of 28–38°C. The temperature coefficient for the above temperature range was 1.5.

It was found that 2,4-D salt concentrations of or exceeding 0.0093 M inhibited the action of wheat lipase. Five trials were made at each of

TABLE I

Comparison of Hydrolysis in the Presence and in the Absence of the Enzyme

Condition	μl. of CO_2/50 min.			
	Run 1	Run 2	Run 3	Run 4
Absence of enzyme	115	65	67	96
Presence of enzyme	236	238	232	239

TABLE II

Effect of Substrate Concentration on Rate of Self-Hydrolysis

Substrate conc.	Rate in μl./min.
0.10 M	1.18
0.15 M	1.70
0.20 M	2.16
0.30 M	2.46

various salt concentrations using 0.3 M glycol monoacetate as the substrate. The reaction rates were calculated in the usual statistical manner. The results of these calculations are expressed in Table III. There was a slight indication of enzyme activation at a salt concentration of 0.0047 M; however, 2 trials were made at salt concentrations of 0.0037 and 0.0019 M, with no demonstrable effect on the activity of the enzyme. An investigation of inhibitor concentrations higher than 0.075 M was not feasible beacuse of the limited solubility of the 2,4-D salt in water.

The type of inhibition by the 2,4-D salt was determined by the method of Lineweaver and Burk (6). The velocity constants were determined over a range of substrate concentrations and at 3 inhibitor concentrations. Four trials were made at each substrate concentration and the best slope calculated. The data for varied substrate concentration and varied inhibitor concentration are listed in Table IV. The reciprocals of the calculated values were plotted as a function of reciprocal substrate concentration in Fig. 1. The straight lines drawn through the points plotted on this graph were determined by statistical methods (5). The calculated slopes and the values obtained from division of the slope by the intercept are listed in Table V. The slope/intercept value of 0.126 M at zero inhibitor concentration is the Michaelis

TABLE III

Effect of the 2,4-D Salt on Rate of Enzymatic Hydrolysis of 0.3 M Glycol Monoacetate

Molar conc. of 2,4-D salt	μl. of CO_2/min.	Percentage of inhibition
0.0000	4.84	0
0.0047	4.91	−1
0.0093	4.64	4
0.0186	4.12	15
0.0372	3.15	35
0.0559	2.40	50
0.0745	1.78	63

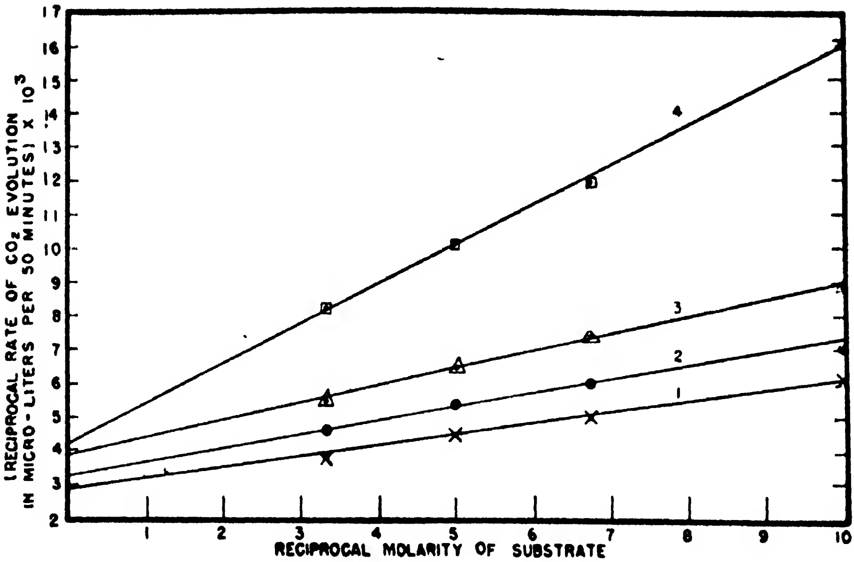

Fig. 1. Type of inhibition of wheat germ lipase by the 2,4-D sodium salt. Salt concentrations are as follows: 1—0.000 *M*; 2—0.019 *M*; 3—0.038 *M*; 4—0.056 *M*.

TABLE IV

Variation of the Substrate Concentration in the Presence of the 2,4-D Salt

Molarity of substrate	µl. of CO_2 evolved/min.			
	0.000 *M* 2,4-D	0.019 *M* 2,4-D	0.037 *M* 2,4-D	0.056 *M* 2,4-D
0.10	3.22	2.81	2.23	1.24
0.15	3.93	3.27	2.67	1.66
0.20	4.39	3.68	3.08	1.97
0.30	5.18	4.32	3.63	2.42

TABLE V

Statistical Values Calculated on the Basis of Fig. 1

2,4-D salt concentrations	Slope in mol. 50-min./liter µl.	Slope/intercept in *M*/l.
0.000 *M*	0.348×10^{-3}	0.126
0.019 *M*	0.413×10^{-3}	0.128
0.037 *M*	0.516×10^{-3}	0.133
0.056 *M*	1.180×10^{-3}	0.276

constant. Table V shows that the slope/intercept values for the first 3 lines on the graph are identical to 2 significant figures. The deviation of the slope/intercept value for the 0.056 *M* inhibitor concentration may be largely due to the probability of greater error in the determination at the 0.10 *M* substrate concentration. This determination is subject to considerable variation since the CO_2 evolution at this concentration is relatively small. It may be observed from consideration of the graph that if this point were ignored, a straight line could readily be fitted to the other 3 points at the 0.056 *M* inhibitor concentration. The resulting line should have a smaller slope and a larger intercept yielding a smaller slope/intercept value. Consideration of Table V and the graph shows that the inhibition is primarily the noncompetitive type, with a tendency toward competitive inhibition at higher 2,4-D salt concentrations.

Discussion

Autohydrolysis of the Substrate

Analysis of the experimental data with respect to the autohydrolysis of the substrate requires careful consideration. It was unexpected that the rates of autohydrolysis of the substrate would be so large, or that they would differ so markedly from one trial to another. The question arose as to whether or not this autohydrolysis occurs at least to such a marked extent in the presence of the enzyme. The experimental data were, therefore, examined critically with respect to this question.

The data given in Table I indicate that the largest rate of autohydrolysis noted was 77% greater than the lowest, as compared with a maximum variation of 3% for the rate of enzymic hydrolysis. Since the latter rates are only from 2 to 4 times as large as the former, if they are of the same magnitude in the presence as in the absence of enzyme, they should result in appreciable variations in the rates of total hydrolysis in the presence of enzyme. Since this is not the case, the absolute variations must not be large in the presence of enzyme, *i.e.*, either the absolute rates of autohydrolysis must be small or, if of the same order of magnitude as in the absence of enzyme, they must be much more nearly equal in the presence of enzyme. The latter possibility is ruled out by the following: If an autohydrolysis correction were made, the two best straight lines for determinations at 0.019 *M* and 0.137 *M* inhibition concentrations would have negative ordinate intercepts, indicating, at high enzyme concentrations, negative reaction rates of substrate

disappearance. This is manifestly impossible in the initial absence of substances required for the synthesis of the substrate.

If the rate of enzymic hydrolysis were corrected for autohydrolysis on the assumption that the latter could be determined by direct measurement in the absence of enzyme, the resulting net rates of enzymic

TABLE VI

Effect of Autohydrolysis Correction on Calculated Energy of Activation

	µl. of CO_2/min.		Energy of activation kg.-cal./mol.
	28°	38°	
Corrected for autohydrolysis	2.89	2.89	0
Not corrected for autohydrolysis	3.37	5.11	8

hydrolysis would not be a function of temperature (Table VI), indicating a zero energy of activation. This result is contrary to generalizations which previously have been made regarding enzymic hydroylsis.

In view of the above considerations the rate of enzymic hydrolysis was not corrected for autohydrolysis.

Relation of the Inhibitive Action of the Sodium Salt of 2,4-D to its Action as a Selective Herbicide

In general, the 2,4-D compounds at low concentrations have a lethal effect upon broad leaf species but do not inhibit the growth of narrow leaf species. This selectivity has been utilized in control of weeds in crop production. The sodium salt of 2,4-D is used in amounts of the order of 1 lb./acre; hence, the amount of 2,4-D that reaches each plant must be very small. Hagen *et al.* (2) have studied the effect of the sodium salt of 2,4-D upon a lipase of a broad leaf plant, *i.e.*, castor bean lipase, using olive oil as a substrate. Completion of the study has made possible a comparison of the effect of the 2,4-D salt on a lipase of a broad leaf plant and that of a narrow leaf plant. A comparison of percentage inhibition was made on a lipase activity basis. This activity basis of comparison was the molar rate of hydrolysis of the substrate under the conditions of optimum pH, optimum temperature, and excess substrate concentrations. On this basis, the sodium salt of 2,4-D was 400 times as effective an inhibitor of the castor bean lipase as the

wheat germ lipase. In other words, the sodium salt of 2,4-D does inhibit an important growth enzyme of a broad leaf plant at relatively low concentrations, but does not effect a comparable enzyme of the narrow leaf plant species until relatively high 2,4-D concentrations are reached. It is conceivable that this is a part of the explanation of the action of the sodium salt of 2,4-D as a selective herbicide.

Summary

A study of the effect of the sodium salt of 2,4-D upon wheat germ lipase is presented. The salt inhibits the action of the lipase at concentrations greater than about 0.009 *M*, under the given experimental conditions. The primary type of inhibition was found to be non-competitive.

A comparison of percentage inhibition on a lipase activity basis was made between the castor bean lipase of the broad leaf family and wheat germ lipase of the narrow leaf family. This calculation indicated the inhibitory action upon castor bean lipase to be of the order of 400 times as effective as upon the wheat germ lipase.

The energy of activation of wheat germ lipase in its action on glycol monoacetate is 8 kg.-cal./mole. The temperature coefficient for the lipase between 28 and 38°C. is 1.5. The value of the Michaelis constant is 0.13 *M*.

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The Mechanism of Action of Phosgene and Diphosgene [1]

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Introduction

The classical view (1–9) that phosgene is a slowly reacting compound which exerts its lethal action by the intracellular liberation of hydrocloric acid does not seem justified on a careful examination of the compound's chemical properties (10). One group of workers (11) indicated that acid liberation may not be the cause of phosgene toxicity but offered no specific substitute. They found (12) that when phosgene in a miscible solvent is reacted with water, the rate of hydrolysis as measured by a colorimetric pH determination is extremely rapid. This has been supported by recent work (13,14). When our own determinations of phosgene disappearance in tissue homogenates (described below) also indicated high reactivity for phosgene, we sought another chemical property which might better explain the compound's lethal action. Such a property is its acyl chloride nature. Since in this property diphosgene behaves exactly like two molecules of phosgene, diphosgene was used in the model compound experiments to facilitate handling.

Phosgene may be considered as the acid chloride of chloroformic acid. It forms a diamide with organic amines in two stages as follows:

$$\mathrm{Cl{-}\overset{\overset{\large O}{/\!/}}{C}{-}Cl + R{-}NH_2 \rightarrow Cl\,\overset{\overset{\large O}{/\!/}}{C}{-\!\!-}\overset{H}{N}{-}R + HCl,} \qquad (1)$$

$$\mathrm{Cl{-}\overset{\overset{\large O}{/\!/}}{C}{-\!\!-}\overset{H}{N}{-}R + R{-}NH_2 \rightarrow R\overset{H}{N}{-}\overset{\overset{\large O}{/\!/}}{C}{-\!\!-}\overset{H}{N}R + HCl.} \qquad (2)$$

[1] This represents experimental work done for the Committee on Therapy of Gas Casualties of the CMR and was summarized in Reports No. 2, April 30, 1942 and No. 7, Feb. 12, 1943, OEM-cmr-114.

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The result is a disubstituted urea.

The reaction with water

$$COCl_2 + 2H_2O \rightarrow CO_2 + 2HCl \quad (3)$$

probably goes in two similar stages. Comparable reactions occur with alcohols and mercaptans.

Most important for its biological significance, the acylation reaction of Eqs. 1 and 2 will take place in the presence of water and often in preference to Eq. 3. The reaction with aniline water is the best example of this (15).

The evaluation of the acylation of biologically significant substances by diphosgene in the presence of water is described below.

If the acylation of tissue substances is the true cause of phosgene toxicity, other volatile acylating agents might be expected to have effects on lung similar to that of phosgene. Such an agent is ketene, $H_2C=C=O$ which has been frequently used to acetylate the free amino groups of proteins in aqueous solution. On hydrolysis ketene gives the weak acid (16) acetic acid. Similarities between phosgene and ketene action have been noted (17).

Rats and yeast suspensions were exposed to ketene and the results given below substantiate the acylation hypothesis.

METHODS

A. Colorimetric Phosgene Determination

This is based on the spot test of Anger and Wang (18).

1. Reagents. One per cent solution of phenyl hydrazine cinnamate in benzene, made up fresh just before the determination; 10% aqueous *p*-methylaminophenol sulfate (metol); solution of phosgene in benzene standardized gravimetrically by diphenylurea method (15).

2. Procedure. Two and one-half grams of lung tissue is homogenized with 25 ml. of Ringer solution in an Elvehjem-Potter homogenizer. One ml. of a benzene solution containing 200 µg. of phosgene is added and the homogenizing process briefly repeated. Fifteen ml. pure benzene is added, the mixture is saturated with salt, and the whole centrifuged at high speed. Two-ml. portions of the benzene supernatant are taken for the determination.

To each unknown solution and to a set of standard solutions containing 5–50 µg. of phosgene is added 2 ml. of the phenylhydrazine reagent. The mixture is let stand 5 min., 0.5 ml. 10% $CaSO_4$ and 0.4 ml. metol solution are added and the mixture is shaken for 2 min. The layers are allowed to settle and the colored benzene phase is read immediately in a photoelectric colorimeter at 520 mµ. A reagent blank is used for zero adjustment.

For the range of 5–50 μg. of phosgene the method is accurate to 1 μg. when timing is carefully controlled.

B. Reaction With Model Compounds

It was desired to determine what proportion of diphosgene used combines with the test compound by reactions analogous to Eqs. 1 and 2 and what proportion combines with water according to Eq. 3. Since only Eq. 3 produces a gaseous reaction product its extent can be measured micromanometrically and Eqs. 1 plus 2 determined by difference. For this measurement the Warburg respirometer was ideally suited.

In the main chamber of a Warburg vessel was pipetted 1.5 ml. of a solution of the compound to be tested, containing 1, 2, or 10 equivalents for 1 mg. of diphosgene. In one side arm was 0.02 ml. Tergitol Penetrant (UCC) to serve as an emulsifying agent; in the other side arm was 0.2 ml. 2 *N* H_2SO_4 to be tipped at the end of the experiment to insure complete CO_2 liberation. After equilibration at 27° or 29°C., 1 mg. of diphosgene dissolved in 0.3 ml. light mineral oil (USP) was added to the Tergitol side arm, stopcocks were closed, and the diphosgene was tipped into the main chamber as soon as convenient. When gas production ceased, the H_2SO_4 was tipped into the main chamber and after a short period of shaking the final reading was taken. The theoretical volume of CO_2 at experimental temperature and pressure from the hydrolysis of 1 mg. diphosgene is 220 mm.[3]; this was checked experimentally. Any difference from this figure represents the amount of diphosgene captured by the test compound. In the cases where thiols were tested, the run was done under a nitrogen atmosphere and the thiol was protected from autoxidation before outgassing by keeping it in acid solution. On tipping, the medium was neutralized. In two cases, cysteine SH was determined by replacing the N_2 with O_2 at the end of the run, adding Fe^{+++} as catalyst and measuring oxygen uptake.

The experiments on yeast fermentation were done on a fresh culture of baker's yeast no more than 48 hr. old. One ml. of the culture was treated with 1 mg. of diphosgene both with and without the substance to be tested, present in 10 times the stoichiometric quantity for the diphogene. The treated suspension was added to 14 ml. 0.1 *M* glucose solution and placed in a fermentation tube. Amount of protection was obtained by comparison of gas production in experimental *vs.* control tubes.

C. Ketene Exposure of Rats and Yeast[3]

Ketene was generated by the pyrolysis of actone in a lamp similar to that described by Williams and Hurd (20). After an even flow had been established, the effluent of the lamp was directed into a small chamber within which were the young (150 g.) albino rats or adult white mice to be exposed. The effluent from this chamber was bubbled through aniline water saturated with acetanilide for a measured period of time during the middle of the exposure. The resultant precipitate of acetanilide was collected, dried, and weighed and from it the average chamber ketene concentration was calculated.

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[3] This exposure was done through the courtesy and in the laboratory of Professor Charles D. Hurd, Northwestern University.

Several suspensions of baker's yeast cultures not over 48 hr. old were exposed by bubbling ketene through them for a measured period of time. Their fermentative activity was measured as in Ref. 21.

The exposed animals were observed for survival. Gross and microscopic pathological findings were obtained on one group of rats and mice. Another group of rats exposed to 0.5 mg./l. for 1.5 min. was sacrificed and a homogenate of lung was tested for enzyme activity by the methylene blue reduction technique described in Ref. 21.

Results

A. The Reaction of Phosgene With Tissue Suspensions

By the time the reagents were added to the tissue-phosgene homogenate (2–3 min.) there was no phosgene detectable. The control tubes gave their usual reaction. Under these conditions the destruction of phosgene is so rapid as to be unmeasurable.

B. Reactions With Model Compounds

The results of reaction with typical compounds is shown in Table I. Fifty-four other substances were tested and their reactions all fell in line with those given.

In the case where SH groups of cysteine were determined after exposure to diphosgene, 80% of the cysteine SH was bound, according to our figures.

C. Ketene Exposure of Rats and Yeast

Ketene kills rats and mice with the same clinical picture as phosgene —*i.e.*, asphyxial death in 0.5–24 hr. with frothy pink-fluid running from mouth and nostrils. The gross and microscopic pathological findings are also identical with those of phosgene poisoning. The sections of the lungs of the ketene animals were given to the pathologist[4] as unknowns and were reported by him "severe phosgene poisoning."

The toxicity of ketene for mice seems some 20 times as great as that of phosgene—7 out of 8 mice exposed to 0.5 mg./l. of ketene for 1.5 min. died.

The effect on enzymatic activity of lung homogenates from exposed animals and on yeast fermentation is shown in Table II.

[4] Dr. C. C. Lushbaugh. He has since extended these observations with others and made additional toxicity studies (19).

TABLE I

Compound	% Diphosgene carbonyl bound — Equivalents of compound: 1	2	10	Binds diphosgene acid	Protection of yeast by 10 equivalents of compound +++ Complete ++ Considerable + Fair 0 None
1. Hexamethylenetetramine	80		88	+	+++
2. Glycine					
pH 10.5 (free base)	30		30		+
pH 7	0		0		
3. Glycine ethyl ester hydrochloride	60		80		++
4. Taurine	50		75	−	+
5. Urea			0		
6. Diethylamine		0		?	
7. Diethanolamine					
neutral		35		−	
free base		55			
8. Aniline					
neutral	70		95	−	
acid salt	10		25		
9. Sulfanilic acid	45		70	?	+
10. *p*-Aminobenzoic acid	65		80		+++
11. 1,2-Diaminophenyl-4-sulfonic acid		80		−	++
12. 2-Napthylamine-6-sulfonic acid		50		−	+++
13. Nicotinamide			0[a]		
14. Thiamine HCl			0[a]		
15. Methylene blue			0		0
16. *p*-Hydrazinobenzoic acid	65+				
17. Cysteine					
hydrochloride			0		
neutral		80			
18. Cystine		0			
19. Glutathione (neutral)		60			
20. Hexyl alcohol		25			
21. Serine		40			
22. Threonine		50			
23. Cholesterol	0				

[a] Accelerates decomposition.

TABLE II

Action of Ketene, Acid, and Diphosgene on Lung Homogenate and Yeast

Material	Measurement	Substrate	% Inhibition			
			Ketene	HAc	HCl	Diphosgene
Lung homogenate	Methylene blue reduction	None	80			70
		Succinate	70	40	35	65
		Glycerophosphate	70		15	40
Baker's yeast	Gas production	Glucose	100	0	0	100

Discussion

The rapid disappearance of phosgene on contact with tissue is entirely consonant with a rapid rate of hydrolysis and indicates that the relative immiscibility of phosgene and water, not the reaction rate, was the limiting factor in most of the earlier work. In the lung with its great area of moist tissue and with phosgene in the gas phase, miscibility need be considered no longer as a factor. It is evident from the work on model compounds that however rapid the hydrolysis, there are some compounds which can compete with water for phosgene and that the functional groups responsible for these reactions are in most instances similar to those occurring in tissue proteins.

The primary amino group, as in aniline, is highly reactive. That this is true of amino acids when the carboxyl group function is suppressed is shown by glycine at pH 10.5 and by glycine ethyl ester. Secondary amines are less reactive as witness the behavior of diethylamine and diethanolamine. Amide groups and amino groups in resonant structures have no reactivity as in the case of urea, nicotinamide, and methylene blue. Mercapto groups are also highly reactive as shown by cysteine. This has been demonstrated by iodine titration by Barron *et al.* (22). The hydroxyl group which also occurs naturally, has a somewhat lower reactivity as shown by hexyl alcohol, serine, and threonine. The hydrazino group, which does not occur naturally, is highly reactive (*p*-hydrazinobenzoic acid) and should be mentioned here in passing. Free amino, hydroxyl, and mercapto groups all occur in tissue proteins

and it seems most likely that acylation of such groups in essential proteins (*i.e.*, enzymes) is responsible for toxic alteration of function.

If this is true, it might be possible to introduce into the living animal before exposure, competing compounds such as those in Table I which would head off enough phosgene at the level of the lung capillary to act as a prophylactic agent against phosgene. Many suitable compounds, such as the aromatic amines, are too toxic for such use, but introduction of sulfonic acid groups lowers toxicity without lowering reactivity to phosgene correspondingly, and 1,2-diaminophenyl-4-sulfonic acid and 2-napthylamine-1-sulfonic acid do give measurable protection in mice (25). Other compounds that give protection in mice presumably on the same basis are hexamethylene tetramine (26) and taurine (25). None of these compounds shows the least protection if given after exposure to phosgene.

As shown in the last column of Table I, the ability of a compound to protect yeast fermentation against diphosgene exposure is correlated not with ability to neutralize acid produced by diphosgene, but with ability to bind the carbonyl in the presence of water. In the work of Winternitz *et al.* (23) and experiments from this laboratory (24) dogs exposed to 0.66 mg./l. of HCl for 30 min. ($\cong$ 0.89 mg./l. phosgene or 9 $\times$ the CT_{50}) showed no ill effects. Just as important, when the HCl dose was raised to the lethal level, distress was immediate and death occurred within 3–5 min. This is not the same pathological entity as phosgene poisoning where with an LC_{50} no effect is visible for 30 min. and death may be delayed 48 hr. or even more. It is well understood that it is possible to administer such massive doses of phosgene that liberated HCl does play a part in lethal action. Significantly, in such animals death is sudden and the pathological picture, dominated by the presence of acid hematin, is qualitatively different from the typical pulmonary edema of phosgene poisoning.

Moreover, as shown above, the phosgene picture is reproduced exactly by ketene, a substance remarkable for its acetylating ability and which gives on hydrolysis only the weak acid, acetic acid. Since ketene is some 20 times as toxic as phosgene weight for weight, it liberates per same lethal dose only 1/48 as many equivalents of this weak acid as phosgene does HCl, making acid production an untenable explanation for ketene toxicity. A third substance which does not acylate but which methylates similar functional groups and which produces no acid whatever is diazomethane. Death from diazomethane is by typical pulmonary edema.

Conclusions

1. The destruction of undiluted phosgene under conditions comparable to those encountered in the mammalian lung occurs in an extremely short time interval (much less than one second).

2. The total hydrolysis of lethal concentrations of phosgene gives rise to innocuous concentrations of hydrogen chloride.

3. Seventy-seven substances have been tested for their ability to be acylated by diphosgene in the presence of a large excess of water, for their ability to bind acid released by diphosgene, and for their ability to protect yeast fermentation against exposure to diphosgene.

4. The ability to be acylated by phosgene in the presence of water depends on the presence of free amino, hydrazino, mercapto, or hydroxyl groups.

5. The protection of yeast fermentation can be correlated with the ability of the substances to be acylated by diphosgene; not with their ability to neutralize liberated acid.

6. Ketene, a substance noted for its acetylating ability and which in effective dosage equal to phosgene liberates insignificant amounts of weak acetic acid, can reproduce exactly the clinical and pathological picture of phosgene poisoning.

7. Because of these considerations and others of a pathological nature, it is extremely unlikely that acid production plays a significant role in the action of ordinarily lethal concentrations of phosgene or diphosgene.

8. Conversely, because of (*a*) the known acylating reactions of phosgene with type compounds, (*b*) the ability of such compounds to protect animals and yeast against phosgene damage, (*c*) the destruction of enzyme systems by phosgene, and (*d*) the ketene and diazomethane analogy, the lethal action of phosgene is concluded to be directly due to inactivation by acylation of essential lung cell constituents.

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The Influence of Buffer Composition, pH and Aggregation on the Thermal Denaturation of Tobacco Mosaic Virus [1]

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Introduction

It was shown several years ago that the rate of denaturation of tobacco mosaic virus (TMV) as measured by precipitation on heating is a first-order reaction (1). Recent studies by Johnson, Baylor and Fraser (2) have indicated that the thermal denaturation is first-order at pressures from 1–680 atm. and that the theory of Absolute Reaction Rates (3) can be applied to describe and predict rates of thermal denaturation at different temperatures and pressures. In extending this work after several months lapse, rates were obtained which differed by nearly an order of magnitude from the previous results. As a preliminary to the study of the effects of drugs and other factors, it was essential to determine that the denaturation rate is a reproducible property of tobacco mosaic virus and also to establish the conditions under which such reproducible rates could be secured. The effect of the major variables on the rate is also fundamental to more complete understanding of the basic kinetics of this reaction.

This paper considers the effect of pH, buffer composition, and aggregation of the virus, demonstrating that the first two are of primary importance. This is consistent with a mechanism involving reaction of TMV with hydroxyl ions in the rate determining step. These findings are a substantiation of predictions made by Lauffer (1) in his original paper.

[1] Aided by a grant from the American Cancer Society through the Committee on Growth of the National Research Council.

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Methods

Denaturation rates of 6 mg./ml. TMV in 0.1 *M* phosphate buffer at 68.8° were determined as described in a previous paper (2). The virus used was either the same ultracentrifugally-isolated stock used previously (but now considerably aggregated after storage in a refrigerator over the summer as a 30 mg./ml. solution in distilled water) or freshly prepared material. The buffers used were prepared with great care from redried Merck anhydrous salts except as otherwise noted. All pH's were measured at 25.0° with a Beckman Model G meter standardized with 0.05 molar potassium acid phthalate, pH 4.00/25.0°. The latter was always freshly prepared from a 0.25 molar solution of redried Mallinckrodt analytical reagent salt. To study the relation of pH to temperature a 3-gallon, well-stirred bath was adjusted to various temperatures with a Variac-controlled Lolag heater. The pH meter was placed so that the 5 ml. beaker was almost completely immersed and the electrodes could be dipped into it as usual. In addition to the above mentioned Merck buffers, a standardizing buffer was made according to Britton (4). At each temperature, successive measurements were made of this buffer, the other buffers, and the usual denaturation mixture. The pH of Britton's buffer is known accurately from 12.5–91°, and hence the difference between Britton's value and our reading at a given temperature constitutes a correction which may be applied to each of the other samples. In this way the difficult problem of temperature compensation of the meter was avoided. The summary of Table II shows that all samples had the same pH at each temperature within the error of this rough experiment.

Results and Discussion

The Effect of pH on the Rate

Lauffer (1) has suggested that the rate of thermal denaturation of tobacco mosaic virus may be proportional to the inverse third power of the hydrogen ion concentration. To examine this more closely, experiments were designed to give the maximum spread of rates consistent with reasonable times of denaturation. To permit running the various pH's simultaneously only one sample was used per pH. The results were treated as one point curves, reasonable log per cent intercepts being assumed from previous comparable experiments (2), and k's calculated as usual. The data (Table I) are plotted (Fig. 1) as $-\log_{10}k$ *vs.* $-\log_{10}$ [H] *i.e.* pH, and the best straight line determined by the method of least squares. The slope of the normal equation of 2.93 ± 0.08 is an excellent check of Lauffer's prediction and the implied relation between k and pH has been used in further work (see Table 4) to correct results at slightly differing pH's to pH 7.00. (The adjustment of the experimental samples to the various pH's required alteration of the ratios of Na_2HPO_4 and KH_2PO_4 and hence the above result should be corrected for the difference in ionic strength as discussed below. The

correction is small but in the proper direction and the final slope is 3 within the 0.01 pH minimum error of the pH meter.)

The burden which this places on the pH determination for each experiment should be stressed. The assumption of a reading error of ± 0.01 pH, a minimum estimate for this type of meter, gives an approximate error of 0.03 in log k and hence in $\Delta V^{\ddagger}$, calculated between atmospheric pressure and 10,000 lb. a possible error of about 6%;

TABLE I

The Effect of pH on the Rate of Denaturation

All experiments at 68.8°

Time	Colorimeter readings	Dilution	%	$k \times 10^3$	pH
Expt. 15*a*					
0	50.9 51.3	10	100		
241	55.7 54.9	9	79	0.85	6.89
190	53.0 52.0 51.3 51.7	7	68	1.82	7.00
190	54.2 54.4	4	36.5	5.05	7.14
60	56.9 54.3 55.9 56.8	5	43	13.6	7.30
Expt. 14 Data below.				3.11	7.07
Expt. 13					
0	52.5 (av. of 7 dtns.)	10	100		
10	55.3 (av. 7)	10	91		
40	53.1 (av. 7)	9	87.5		
130	55.0 (av. 5)	7	64.8		
255	55.6 (av. 5)	5	45.4		
				2.84	7.05

between atmospheric pressure and 5000 lb. an error of about 11%. This error is inherent in the experiment and is in addition to any error in sampling and Kjeldahl, etc. To reduce this deviation to a minimum it is necessary to check the virus solution and all other solutions being added to buffer for neutrality before mixing and to perform critical experiments either simultaneously or as rapidly in succession as possible. For most consistent results, a single stock of buffer obviously should be used for an entire series of experiments and the buffer used to standardize the pH meter should be most accurately reproducible. The temperature of the solutions must be taken as the pH is measured

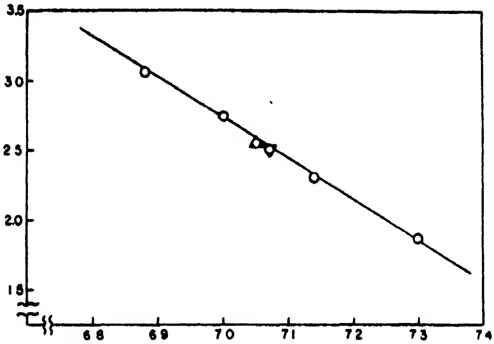

FIG. 1. Minus $\log_{10}$ of the reaction constant against pH. The least squares line through these points has a slope of 2.93. In addition to the points from a single experiment (15*a*), two other complete experiments (13, triangle, and 14, inverted triangle) are included. The data are from Table I.

and we have found it necessary to adjust all solutions to 25.0° in order secure satisfactory accuracy.

Another factor of considerable importance in pH, is that of the dilution of the buffer in making up experimental mixtures. A 0.2 *M* phosphate buffer, for example, becomes more alkaline by approximately

TABLE II

The Effect of Temperature on the pH of the Reaction

Solution	pH at a given temperature				
Britton std. buffer	6.96/25.0	7.10/39.0	7.21/48.5	7.35/58.0	7.51/69.0
Usual buffer+virus	7.02/25.0	7.12/39.0	7.25/48.5	7.39/57.0	7.52/67.0
0.2 *M* buffer	6.95/25.0	7.10/39.0	7.21/48.5	7.38/58.7	7.43/65.0
0.1 *M* buffer	7.00/25.0	7.14/39.0	7.21/49.0	7.41/58.3	7.48/67.0
0.01 *M* buffer	7.02/25.0	7.14/39.0	7.28/49.2	7.42/58.0	7.57/67.0

Summary of corrections

	25°	39°	48–49°	57–59°	65–70°
Britton std.	6.96	0[a]	0[a]	0[a]	0[a]
Buffer+virus	6.98	0	+0.02	+0.02	−0.01
0.2 *M* buffer	6.95	+0.01	+0.01	+0.04	0
0.1 *M* buffer	7.00	0	−0.05	+0.03	−0.04
0.01 *M* buffer	7.02	−0.02	0	+0.02	+0.03

[a] See ref. 4.

0.05–0.06 pH at pH 7 when diluted with an equal volume of distilled water or virus solution. Hence all buffers must be so made that the correct pH is reached *after* final mixing and all solutions must be rechecked at this stage.

The Effect of Temperature on the pH

Since pH is so important a factor in the reaction rate, it was essential to know if the pH of the denaturing solution were reasonably invariant with temperature. Obviously, calculations of the various thermodynamic parameters (2) are meaningless if the differences in rate at various temperatures are largely ascribable to pH changes. Comparison by the method described above of various strengths of buffer and of an actual sample reaction mixture with a buffer whose pH *vs.* *T* dependence is known showed (Table II) that within the limits of this necessarily rough experiment the pH is constant at pH 7. The errors are random, and the possibility of an important rate change due to the effect of temperature on pH has been excluded.

We have also shown that under the stated conditions the pH does not change measurably during the denaturation process.

The Effect of Buffer Concentration on the Rate (Salt Effect)

Aside from the effect on pH during dilution, moreover, buffer concentration is itself a critical factor because of the change in ionic

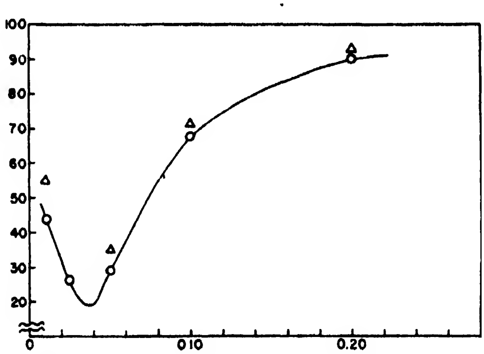

FIG. 2. Per cent TMV undenatured in 190 min. against the molarity of the buffer in which the reaction was run. The upper points (triangles) are from an experiment with aggregated virus, the lower points (circles) from a similar experiment with fresh virus.

strength. Two experiments, one with aggregated and another with fresh virus, showed a considerable increase in rate as the buffer concentration went from 0.2 to 0.1 to 0.05 *M*. This effect, dependent on the effect of ionic strength on activities, is followed by a sharp *decrease* in rate at 0.01 *M* (Fig. 2). It is believed that the apparent decrease in the rate at the lowest concentration of buffer is largely due to slower coagulation of the denatured virus. Schachman (5) has found that the aggregation of the virus during preparation also depends on the buffer concentration and that a sharp decrease in rate of aggregation occurs in the same range of buffer strength. The two phenomena are probably related. The importance of buffer concentration is as great as that of pH over the range studied, but is more easily controlled by using a single buffer stock for an entire series of experiments.

The relation of the salt effect to the kinetics is discussed below.

The Effect of Buffer Composition on the Rate

Despite the necessity demonstrated above for using anhydrous salts, an experiment was performed attempting to duplicate the previous results (2) by using Baker salts, KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$. At 0.1 *M* the rate with the Baker salts was very slow, almost negligible, in 3½ hr., whereas, the control (usual Merck buffer) showed a normal rate. Even granting partial dehydration of the $Na_2HPO_4 \cdot 12H_2O$ the difference is difficult to explain. It is possible that minor differences in trace impurities, possibly ions such as Ca or Mg, may be of importance.

Since the ratio of sodium to potassium is varied to adjust the pH for individual experiments it was questioned whether this could affect the rate, but no significant difference was found when potassium salts only were used in the buffer.

Reproducibility of the Rate with Fresh and Aggregated Virus Preparations

Lauffer (1) found that samples which he had isolated by chemical or ultracentrifugal techniques did not differ greatly even when presumably aggregated material was compared with fresh. His greatest difference was 8.7×10^{-3} *vs.* 13.5×10^{-3}/min. at 69°. Except where noted we have used the ultracentrifugally-isolated virus of our previous paper (2) but now strongly aggregated. Under as closely controlled conditions as we have been able to attain, we still find slopes appreciably different

TABLE III

Effect of Buffer Concentration

Time	Colorimeter readings			Dilution	%	Buffer concn.	% in 190 min.
Expt. 15*b* Fresh virus 5.92 mg./ml. pH 7.00/25°							
0		53.7	53.8	10	100	0.2	
0		50.9	51.3	10	100	0.1	
0		50.9	51.3	10	100	0.01	
	Zero used						
190	5.45	53.9		9	90	0.2	
190	5.92	52.0 (av. of 4)		7	68	0.1	
190	5.92	52.0	50.9	3	29.5	0.5	
190	5.90	63.8	63.7	4	26.5	0.025	
190	5.90	26.3	25.8	2.2	44	0.01	
		56.2	55.2	5			
Expt. 12 Aggregated virus *ca.* 6 mg./ml. pH 6.99–7.00							
a. 0.2 *M*							
0		54.0	55.0	10	100		
10		52.4	52.8	9.5	100		
30		50.8	50.8	9	100		
120		51.9	53.2	9	95		
180			53.1	9	93		
						0.2	93
b. 0.1 *M*							
0		54.8	54.7	10	100		
10		56.6	56.2	10	97		
60		52.1	52.6	8	89		
150		53.5	53.6	7	75		
237		51.0	53.3	6	67		
						0.1	71
c. 0.05 *M*							
0		56.2 (av. of 6)		10	100		
10		53.8 (av. of 4)		9	97		
20		53.9	55.3	9	89		
45		57.4 (av. of 4)		8	77		
80		54.0 (av. of 4)		6	64		
						0.05	35
d. 0.01 *M*							
0		52.4 (av. of 3)		10	100		
10		54.6 (av. of 3)		10	94		
20		53.7 (av. of 4)		10	94.5		
45		54.2	54.4	9	84.5		
120		61.1	61.9	9	68		
						0.01	55

from those reported in our last paper (2) and by Lauffer (1). These are compared in Table IV, which shows the present results to be reasonably self-consistent, as was each of the other sets. Further evidence of reproducibility under constant conditions is afforded by the two extra points, Expts. 13 and 14, inserted into Fig. 1.

The parallelism between fresh and aggregated virus noted in the buffer concentration experiments (Fig. 2) indicates that under the same conditions no great discrepancies result from ordinary changes in the virus due to preparation techniques or storage. It can also be seen in Table IV that there is a tendency, previously noted by Lauffer, for preparations to differ and for fresh virus to denature slightly faster than aggregated. It seems likely, however, from current work by Schachman (10) that in 0.1 *M* buffer, as used in the present experiments, the fresh virus may aggregate so rapidly that it is essentially the same as older preparations.

In summary, it may be concluded that under a given set of conditions the denaturation of tobacco mosaic virus is a reproducible characteristic of the molecule. The rate, like other properties, is somewhat dependent on the method of preparation and/or the degree of aggregation. The reaction is, however, so extremely sensitive to changes in buffer concentration and pH that even fairly large discrepancies between results obtained by different observers may be discounted. *To be strictly comparable experiments must be run essentially simultaneously using identical buffers.*

Interpretation of the Results in Terms of Kinetics

The apparent contradiction of saying that the rate is first-order and at the same time dependent on the inverse third power of the hydrogen-ion concentration is, of course, explained by the fact that hydrogen ions or hydroxyl ions are in instantaneous equilibrium with a relatively infinite reservoir of un-ionized water. The concentrations of such ions do not, therefore, change during the reaction and hence appear as constants in the kinetic expression at constant pH. Thus the reaction may indeed be third-order on hydroxyl ions but pseudo first-order on tobacco mosaic virus at each pH.

The presumption that three hydrogen or hydroxyl ions are involved in the rate determining process can be strengthened by a further analysis of the data. Since the rate varies with buffer concentration, the

TABLE IV

Reproducibility of the Rate at 68.8°

Aggregated, recentrifuged virus

Expt.	pH	$k \times 10^3$ calcd.	$k \times 10^3$ corrd. to pH 7.00
10	7.02	1.77	1.54
12	6.99	1.66	1.78
14	7.07	2.26	1.40
17	7.03	1.91	1.55
			1.57 ±0.16 std. deviation
Fresh, ultracentrifugally-isolated virus Sample No. 1			
13	7.05	2.84	2.00
14	7.07	3.11	1.93
15	7.00	1.82	1.82
			1.92 ±0.10
Sample No. 2			
16	7.01	2.81	2.63
22	7.03	3.02	2.46
			2.54 ±0.11
Results reported by Johnson, Baylor and Fraser (2) at 68.8°			
	7.05	7.59	5.37
Results obtained by Lauffer and Price (1) at 69.0°			
Stored virus			
	7.05	8.74	6.19
Fresh virus			
	7.05	13.5	9.56

rate-determining step must involve an ionic reaction. Starting with the form of the Brønsted relationship (6)

$$\log k = \log k_0 + \log \frac{f_a f_b}{f_x}$$

where f_a and f_b are the activities of the reactants, f_x the activity of the activated complex, and k_0 the rate constant at infinite dilution, one may

apply the simplified form of the Debye-Hückel equation

$$- \log f = 0.5\, z^2 \sqrt{\mu}$$

where μ is the ionic strength of the solution and z the charge on the ion, to obtain the following relationship:

$$\log \frac{k}{k_0} = \log \frac{f_{OH} \cdot f_{virus}}{f_{act.\ complex}} = -\,0.5 \sqrt{\mu}\,[3(z_{OH})^2 + (z_v)^2 - (3z_{OH} - z_v{}^2]$$

whence

$$\log \frac{k}{k_0} = \sqrt{\mu} \cdot 3z_{OH}\,(z_v - z_{OH})$$

Here z_{OH} is minus one, and the slope of a plot of log k against the square root of the ionic strength of the solutions from 0.025–0.2 M in buffer is minus three (Fig. 3), well within the error of the experiments. Although the salt effect—particularly in protein denaturations—is a complicated phenomenon, this is consistent with a charge on the attacked centers of the virus of zero and with three hydroxyls being involved in the rate-determining process. It may be of interest to note that it has been found that the more rapid thermal denaturation of

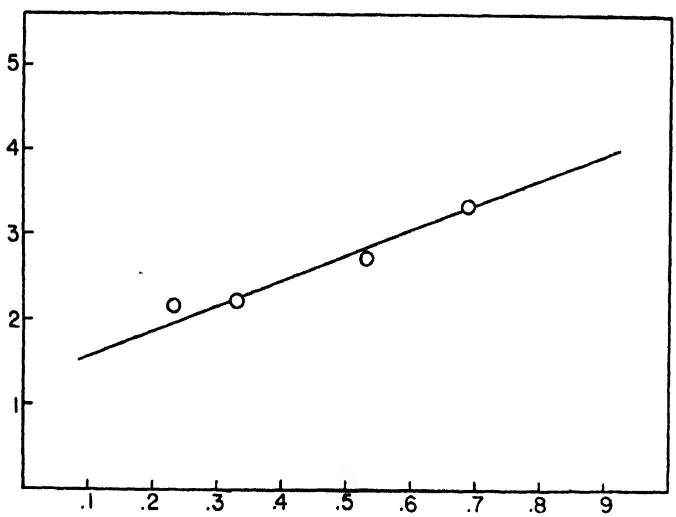

FIG. 3. Minus $\log_{10}$ reaction constant against the square root of μ, the ionic strength. A line of slope 3.0 has been arbitrarily drawn through the points to illustrate the fit of this slope.

the virus with urethan approaches a terminal state in which three urethans are probably involved (8).

The net overall rate-determining reaction is then

$$TMV + 3(OH)^- \longrightarrow TMV_{denatured}\ (ppt.)$$

Steinhardt (7) has found that the inactivation of crystalline pepsin involves five hydroxyls, and this, as well as the pH sensitivity of TMV denaturation, is most readily explained by the assumption (1) that denaturation involves bond rupture accompanied by proton ionization. Hence

$$\begin{aligned} TMV + (OH)^- &\rightleftarrows (TMV)^- + H_2O \\ (TMV)^- + (OH)^- &\rightleftarrows (TMV)^{=} + H_2O \\ (TMV)^{=} + (OH)^- &\rightleftarrows (TMV)^{\equiv} + H_2O \\ (TMV)^{\equiv} &\longrightarrow TMV_{denatured}\ (ppt.) \end{aligned}$$

In this series the rate-determining process is presumably the last step, dissociation of the ionized complex. The fact that several steps precede denaturation as measured by precipitation is interesting in connection with the observation that the rate of virus infectivity loss is also a first-order reaction (9) but (1) a faster one (4–7 times) than the rate of denaturation here measured. It is possible, for example, that infectivity loss is caused by the reaction with only one hydroxyl, or possibly two, although, of course, there may be still other as yet unknown steps in the denaturation process. A study of the effect of pH on infectivity loss would elucidate this point.

It should, perhaps, be pointed out that no implication can be made that the process of precipitation of TMV is completely accomplished by the above steps. Further steps of the same or entirely different nature may be involved with the sole proviso that they be faster than the above rate-determining steps.

Summary

In a search for the explanation of considerable discrepancies in the rate of denaturation of tobacco mosaic virus as measured by precipitation, the effects of the major variables were studied. The important factors were shown to be pH and the concentration of the buffer. It is also possible that minor impurities in the buffer salts may be of importance.

The rate was found to be dependent on the third power of the hydroxyl ion concentration and the inverse third power of the square root

of ionic strength, and the probable kinetics of the reaction are discussed in terms of these factors. Rate discrepancies are probably due to the extreme sensitivity to these variables.

The effect of temperature on the pH of the buffered system was shown to be insufficient to affect the rate seriously.

The age of the virus preparation was found to be a significant but less important factor.

Acknowledgment

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Concentration of the Unidentified Growth Factor in Condensed Fish Solubles [1]

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Introduction

Recent studies indicate that condensed fish solubles (1) contain an unidentified factor or factors which stimulates growth in chicks (2–6) and affect hatchability of eggs (7–8). Apparently the chick-growth factor is distinct from vitamin A, vitamin D, *p*-aminobenzoic acid, biotin, choline, folic acid, inositol, niacin, pantothenic acid, pyridoxine, riboflavin, thiamine, and 2-methylnaphthoquinone (6). Berry *et al.* (2) found that an ethanol extract contained most of the activity. Additional properties have been reported by other workers (9,10) who observed that the chick-growth factor is soluble in water, in 70% methanol, and in 70% ethanol, is somewhat soluble in absolute methanol, is very slightly soluble in 95% ethanol, is insoluble in ether and in acetone, is dialyzable and is stable to heating and to enzymatic digestion. It has also been observed (11,12) that rats respond to additions of this product to vegetable protein diets. This paper describes methods for preparing highly potent concentrates from condensed fish solubles, and the testing of their biological activities with rats.

Experimental

Fractionation of Condensed Fish Solubles

Commercial samples of condensed fish solubles (CFS) from two sources were used as starting material. The products carried 50% solids and were equally satisfactory for fractionation.

[1] Journal Paper No. 399 of the Purdue University Agricultural Experiment Station.

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A 500-g. sample of CFS was shaken with 2 l. of 95% ethanol and the suspension was centrifuged. The residue was successively extracted in similar fashion with 2 l. of 95% ethanol, 1.5 l. of 95% ethanol, 1 l. of 95% ethanol and 1 l. of 99.5% ethanol. The final residue was air-dried and designated as fraction I.

The centrifugates were separately concentrated under reduced pressure and treated with 99.5% ethanol, followed by centrifugation and concentration, until 5% or less water remained. The pooled alcohol precipitates were air-dried and designated as fraction II.

The alcohol extracts were concentrated separately to thick syrups and taken up in distilled water. Lipide material formed a separate hyperphase and was removed by

TABLE I

Composition of Experimental Diets

Ingredient	A	B	C
g./100 g. of diet			
Expeller soybean meal[a]	50.0		
Autoclaved soybean protein[b]		20.0	
Extracted casein[c]			20.0
Distillers' dried solubles	5.0	5.0	
McCullum-Davis salt mixture 185	3.0	4.0	4.0
Cottonseed oil		3.5	
Hydrogenated cottenseed oil	11.5		5.0
Cod liver oil (2800 A, 255 D)	0.5	0.5	[d]
Celluflour		2.0	2.0
Dextrin	30.0		
Cerelose		65.0	68.5
Sulfasuxidine			0.5
mg./100 g. of diet			
Thiamine hydrochloride	0.5	0.75	0.67
Riboflavin	0.5	1.0	0.67
Niacin	5.0	4.2	6.7
Calcium pantothenate	1.0	1.7	1.1
Pyridoxine hydrochloride	1.0	1.7	1.1
p-Aminobenzoic acid	5.0	7.5	11.0
Choline chloride	150.0	170.0	220.0
i-Inositol	50.0	34.0	44.0
Biotin	0.02	0.03	0.04
Folic acid (Lederle)	0.02	0.03	0.04
Menadione	0.10	0.40	0.50

[a] Commercial sample.

[b] Archer-Daniels-Midland No. 36 Powder, treated as described by WESTFALL AND HAUGE, *J. Nutrition* **35**, 379 (1948).

[c] Commercial casein successively extracted with ethyl ether, cold 95% ethanol, and hot 95% ethanol.

[d] CLO one drop every other day, vitamins daily in 1 ml. 50% ethanol.

TABLE II

Biological Tests of Fractions

Basal diet	Kind of supplement	Amount of supplement	Number of rats	Av. gain
		mg./100 g. diet		*g./week*
A	None		8	31.3
	None[a]		8	31.7
	CFS (dry basis)	1000	8	36.4[d]
A[b]	None		10	28.7
	Fraction I	350	10	29.3
	Fraction II	50	10	28.0
	Fraction III	100	10	37.0[d]
	CFS (dry basis)	1000	10	37.4[d]
A[b, c]	None		8	33.3
	Fraction III	100	8	37.7[d]
	CFS (dry basis)	1000	8	39.0[d]
B	None		6	23.8
	Fraction III	100	6	30.6[d]
	CFS (dry basis)	1000	6	32.0[d]
C	None[a]		6	19.2
	Fraction III[a]	100	6	25.3[d]

[a] These animals received water-soluble vitamins daily in solution; all others had these vitamins mixed directly in the ration.

[b] Biotin and folic acid were unavailable for these tests.

[c] DL-Methionine added to all 3 diets (200 mg./g. diet).

[d] Highly significant response over negative control (P = 0.01).

filtration. The aqueous filtrates were clarified with Supercel, pooled, adjusted to pH 4.6, made to 500 ml., and preserved under refrigeration as fraction III. Solid matter which separated on prolonged cold storage carried relatively little activity and was removed by filtration.

The biological activities of the fish solubles and the three fractions were ascertained by observing the growth response of rats during a 21-day test period. Preliminary feeding experiments led to the selection of three basal diets for the growth tests. Composition of these diets is given in Table I.

Weanling albino rats from the Purdue colony (19–22 days, 40–45 g.) were distributed among the test lots with the same number of each sex

in each lot. The animals were housed individually in screen-bottom wire cages and were supplied with food and water *ad lib.* Weight and food-intake were recorded weekly.

Stock litters were used in all tests except that on diet *C*. In the latter case, litters were specially prepared by feeding diet *C* (sulfasuxidine omitted) to the dams starting on the third day following parturition. This procedure caused no observable difference in the weanling young as compared with stock litters.

Supplements tested on diets *A* and *B* were incorporated directly in the ration. In the test on diet *C*, all animals received the basal diet throughout, rats being paired at two weeks on the basis of weight gained, half then receiving a daily supplement during the subsequent test period.

The results of the tests (Table II) show that condensed fish solubles contain a rat-growth factor which is distinct from the known vitamins and methionine. It is apparent that the removal of impurities resulted in about 10-fold concentration of activity.

Some Properties of the Growth Factor

When it was found that the active principle was concentrated in fraction III, a series of similar preparations was made by the same procedure except that the initial extraction was made by stirring fish solubles with 2 volumes 1:1 acetone-ethanol and filtering the suspension. These preparations, designated as 47*A*, 47*B*, and 47*C*, contained the equivalent of 5 g. CFS/ml.

Prepns. 48, 49, 50. A 100-ml. portion of 47*B* was extracted 15 times with equal volumes of chloroform. The pooled extracts were cleared over anhydrous sodium sulfate, freed of chloroform under reduced pressure and dissolved in dilute ethanol (Prepn. 48). The water phase remaining after extraction was similarly freed of chloroform and was preserved as Prepn. 49. Half of 49 was passed through a water-washed 2 × 10 cm. column of 60-mesh Florisil. The filtrate was Prepn. 50.

Prepns. 51, 52. A 100-ml. portion of 47*B* was passed through a water-washed 3 × 15 cm. column of 1:1 fuller's earth-Celite. The filtrate was Prepn. 51. Half of Prepn. 51 was treated with 10 ml. of 50% lead nitrate solution and filtered. The filtrate was saturated with hydrogen sulfide, filtered, neutralized to litmus, again saturated with hydrogen sulfide, and filtered. The fitrate was concentrated under reduced pressure to a syrupy residue from which Prepn. 52 was obtained by extraction with 99.5% ethanol.

Prepn. 54, 55. A 50-g. portion of CFS was stirred with 60 ml. of 1:1 acetone-ethanol and filtered. The filtrate was shaken with an equal volume of chloroform, the hypophase being taken directly as Prepn. 54, the hyperphase as Prepn. 55.

Prepn. 57. A 10-ml. portion of 47*C* was diluted to 40 ml. with distilled water, stirred 15 min. with 10 g. of 60-mesh Florisil and filtered. The filtrate was preserved as Prepn. 57.

Prepn. 58. A 10-ml. portion of 47*C* was diluted to 25 ml. with distilled water, stirred 15 min. with 5 g. of 60-mesh Florisil and filtered. The residue was twice washed with 10 ml. portions of distilled water and was then eluted with 25 ml. of 50% ethanol containing 0.25 ml. of glacial acetic acid. The eluate was preserved as Prepn. 58.

Prepns. 64, 65. A 20-ml. portion of 47*C* was diluted to 50 ml. with distilled water and passed through a water-washed 2 × 10 cm. column of 1:1 Florisil-Supercel. The column was washed succesisvely with 30 ml. of distilled water, 80 ml. of 99.5% ethanol, and 40 ml. of 50% ethanol containing 2 ml. of concentrated ammonium hydroxide. The 99.5% ethanol washings were collected as Prepn. 64, the 50% ethanol washings as Prepn. 65.

Prepn. 66. A 150-ml. portion of 47*C* was filtered through fuller's earth as described in the procedure for Prepn. 51 and was preserved as Prepn. 66.

Prepn. 72. A 60-ml. portion of Prepn. 66 was extracted 5 times with ethyl ether. The water phase was freed of ether on the steam table and preserved as Prepn. 72.

On the basis of preliminary experiments, diet *B* was selected for testing the activity of the various preparations. Stock rats were used throughout. To obtain the greatest amount of information from the least amount of material, the animals were paired at distribution on the basis of sex, litter, and initial weight. One rat of each pair received the basal diet, its mate the test diet.

All fractions were fed at 1–5 equivalent % (g. CFS, dry basis, /100 g. of diet) and were incorporated directly in the test diets. Activity of test material was expressed as per cent increase in 21-day gain of the test rat over its paired control.

The results (Table III) show the efficacy of the described procedure for the preparation of an initial extract and suggest certain properties of the rat-growth factor. Under the experimental conditions imposed, it is evident that the active principle is not extractable with chloroform or with ether, that it is not adsorbed by fuller's earth but is readily adsorbed by and eluted from Florisil, and that it is not precipitated by lead.

Concentration of the Unidentified Growth Factor

On the basis of preliminary experiments a procedure was developed for the concentration of the unidentified growth factor in condensed fish solubles. The method described for Prepn. 47 was used to obtain an extract (47*D*) from 22.5 kg. of CFS. Each ml. of 47*D* represented 50 g. of starting material.

Prepn. 89. A 350-ml. portion of 47*D* was diluted to 800 ml. with distilled water and extracted 5 times with equal volumes of ethyl ether. Dissolved ether was removed from the water phase by evaporation under reduced pressure, and distilled water was added to bring the volume to 1200 ml. Slow addition of 300 ml. of 50% lead nitrate solution

produced a cream-colored precipitate which was removed by filtration. The filtrate was saturated with hydrogen sulfide, filtered, adjusted to pH 7.5, and again saturated with hydrogen sulfide and filtered. The filtrate was freed of alcohol-insoluble material by repeated concentration and extraction with 99.5% ethanol. The alcohol-soluble fraction was then reduced to a thick syrup, diluted with 200 ml. of distilled water, and extracted five times with equal volumes of chloroform. The water phase was freed of chloroform on the steam table to yield 220 ml. of Prepn. 89.

Prepn. 91. A 10-ml. portion of Prepn. 89 was diluted to 100 ml. with distilled water and passed through a 2 × 10 cm. column of 1:1 200 mesh Florisil-Supercel. Without being allowed to run dry, the column was washed with five 10 ml. portions of distilled water. Development with 80% ethanol caused separation of 3 yellow bands, the most rapidly moving of these being completely washed through to yield 30 ml. of the eluate, Prepn. 91.

TABLE III

Some Properties of the Growth Factor

Prepn. tested		Number of rat pairs in test	Activity[a] of prepn.
Number	Description		
47*A*	Alcohol extract	6	28.6
47*B*	Alcohol extract	8	25.7
47*C*	Alcohol extract	6	27.3
48	Chloroform extract	10	2.3
54	Chloroform extract	4	0.0
49	Chloroform-insoluble	10	11.9
55	Chloroform-insoluble	4	14.9
72*A*	Ether-insoluble	4	21.5
72*B*	Ether-insoluble	6	33.2
51	Fuller's earth filtrate	6	13.0
66	Fuller's earth filtrate	4	16.8
50	Florisil filtrate	10	3.2
57	Florisil filtrate	6	9.1
58	Florisil eluate	6	17.0
64	Florisil eluate	4	11.4
65	Florisil eluate	3	15.0
52	Lead filtrate	6	18.8

[a] Average per cent increase of gain made by test rat over paired control.

TABLE IV

Relative Potency of Concentrates

Preparation tested		Number of rat pairs in test	Activity[a] of prepn.
Number	Amount fed		
	mg./100 g. diet		
Condensed fish solubles	1000	8	31.7
47*D*	100	6	26.9
89	20	6	30.6
91[b]	0.8	6	24.8
91	0.2	6	25.4

[a] Average per cent increase of gain made by test rat over paired control.
[b] DL-Methionine added to both diets (150 mg./100 g. diet).

The relative potencies of CFS, preparations 47*D*, 89, and 91 were measured by the growth response of rats, using the technique previously described.

The results (Table IV) demonstrate that the described procedure resulted in concentration of the unidentified growth factor of condensed fish solubles with several thousand-fold increase in potency.

Discussion

Since the test diets used in this study were designed to provide ample allowances of the known vitamins, it is evident that the growth factor of condensed fish solubles is distinct from any of these substances. It is also unlikely that any of the known amino acids could be responsible for the growth-stimulating effect in view of the high potency of Prepn. 91. This concentrate was found to possess appreciable activity at a level of 2 p.p.m. in the diet, a much smaller amount than would be required to demonstrate significant amino acid supplementation.

The ultraviolet absorption spectrum of aqueous solutions of Prepn. 91 showed no characteristic maximum in the region measurable with the Beckman spectrophotometer, exhibiting only a progressively increasing absorption toward the shorter wavelengths. This is in distinct contrast to the absorption curve of vitamin B_{13} (13). Another distinction is the solubility of vitamin B_{13} in ether and in chloroform. Furthermore, inclusion of 5% distiller's dried solubles in the test diet provided ample B_{13} to meet rat needs (14). Therefore, it may be concluded that vita-

min B_{13} and the fish solubles factor are physically and biologically distinct.

There is some evidence that the factor concentrated from condensed fish solubles may be related to other growth factors. Zoöpherin, reported to be identical with factor X and with the cow-manure factor (12), has been found to occur in condensed fish solubles, to show rat-growth activity, and to possess properties similar to those of the growth factor described in the present study. This factor and zoöpherin are both soluble in water and in alcohol, but insoluble in ether, and neither is precipitated by lead at pH 7.5. Although the testing procedures were different, it is possible that the growth factor here described is responsible for the activity of zoöpherin.

It is probable that the growth factor concentrate from condensed fish solubles is a growth-stimulating factor for the chick. Prepn. 47*A* has been found to possess highly significant chick-growth activity (15). A recent report (10) that the chick-growth factor in condensed fish solubles is soluble in water and in alcohol, but is insoluble in ether, affords further evidence favoring identity of the rat and chick factors.

Since crystalline vitamin B_{12} was not available when this work was done, the relationship of this factor to vitamin B_{12} (16) was not established.

Summary

1. Condensed fish solubles contain an unidentified rat-growth factor distinct from the chemically defined vitamins and amino acids.

2. The factor is distinct from vitamin B_{13} and unidentified growth substances in distiller's dried solubles.

3. The factor is soluble in water, in ethanol, and in mixtures of acetone, ethanol, and water, is insoluble in ether and in chloroform, is not adsorbed by fuller's earth, is readily adsorbed by and eluted from Florisil, and is not precipitated by lead.

4. A concentrate of the factor has been obtained which is active at a level of 2 p.p.m. in the diet.

5. This factor is probably responsible for the growth-stimulating effect of condensed fish solubles upon rats and chicks.

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Studies on the Cyclophorase System. XII. Incorporation of P^{32}

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Introduction

The present communication deals with the process by which inorganic phosphate becomes incorporated in or bound to the particulate elements of the cyclophorase system during active oxidation, and is a sequel to a previous study on oxidative phosphorylation by Cross *et al.* (1).

Experimental

Plan of Experiments

The cyclophorase gel of rabbit kidney or liver was allowed to carry on active oxidation in the presence of radioactive phosphate. At the termination of the run the enzyme gel was washed twice with some 50 volumes of cold 0.9% potassium chloride solution. The centrifuged, washed gel was then dissolved in strong potassium hydroxide solution and the radioactivity of the resulting solution was measured in a Geiger-Mueller counter. A blank identical in all respects (except without added substrate) was carried through simultaneously. The ratio of radioactivity in experimental and blank provides some measure of the incorporation of radioactive P directly attributable to the oxidation process under study. It is to be noted that in this type of experiment all soluble components of the enzyme system are washed away and the residual radioactivity of the washed residue can be attributed only to components either bound or adsorbed by the gel. The radioactivity of the blank at 38°C. provides a measure of unspecific adsorption of radioactive phosphate by the gel. The blank is admittedly not a perfect blank since there is always a small residue of active oxidation which undoubtedly contributes to the radioactivity of the blank gel.

A quantitative index of P^{32} incorporation is provided by the quotient: radioactivity per μ-atom of inorganic[2] P in the gel over radioactivity per μ-atom of inorganic P in the medium in which the gel was originally suspended.

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[2] Later in the text there will be amplification of what is meant by inorganic phosphate in the gel.

Incorporation of P^{31} and P^{32} in Cyclophorase Gel

When liver or kidney gel at the third residue stage is shaken in oxygen in the presence of radioactive phosphate it is found that the gel which has been incubated in the presence of substrate (experimental) contains from 3 to 5 times as much radioactivity as the gel which has been incubated without added substrate (blank). Similarly the experimental gel (following treatment with trichloroacetic acid) releases from 3 to 4 times as much inorganic phosphate as does the blank (*cf.* Table I). The same results obtain equally well at 0°C. as at 38°C.

The incorporation of P^{31} and P^{32} have been found to run parallel in all our experiments (except under special conditions to be specified later) and these may be regarded as related processes. The radioactivity of the gel is associated largely with the one or more substances which estimate as inorganic phosphate in the trichloroacetic acid filtrates of the gel.

TABLE I

Incorporation of Inorganic P^{31} and P^{32} in Cyclophorase Gel

	Oxygen absorbed	Inorganic P in washed gel	Radioactivity in washed gel
	μ-atoms	*μ-moles*	*counts/min.*
Kidney cyclophorase—38°C.			
Blank	1	0.35	298
Experimental	42	1.43	1550
Liver cyclophorase—38°C.			
Blank	0.5	0.26	550
Experimental	36.2	0.86	2747
Liver cyclophorase—0°C.			
Blank	—	0.37	820
Experimental	—	1.01	2610

The manometer vessels contained 1.5 ml. of kidney or liver cyclophorase at the third residue stage (R_3K or R_3L), 0.3 ml. of 0.1 *M* phosphate buffer of pH 7.3, 0.3 ml. of 0.01 *M* adenosine-5-phosphate, 0.2 ml. of 0.02 *M* magnesium chloride and water to make a final volume of 3.0 ml. The experimental vessels also contained 0.2 ml. of 0.1 *M* α-ketoglutarate. Alkali was present in the center well. The gas space was filled with oxygen and the vessels were equilibrated in a water bath at 38°C. Oxygen uptake for the first 5 min. was assumed to be equal to that observed the following 5 min. After 15 min. incubation the flasks were chilled and the contents were washed into a centrifuge tube and the gel was washed 2 times with 50 ml. of 0.9% potassium chloride at 0°C. For estimation of inorganic phosphate the gel was mixed with 1 ml. of 50% trichloroacetic acid and water to a volume of 10 ml. and the filtered extract was used directly. Radioactivity measurements were made on 1 ml. of this extract or 1 ml. of a solution of the gel in 5 ml. of 8% potassium hydroxide. The background count in all experiments was between 25 and 35 counts/min. The experiments at 0°C. were carried out for 20 min. in test tubes immersed in an ice bath. The radioactive phosphate solution added initially to each manometer cup had an activity of about 80,000 counts/min.

It can be shown that the substance or substances estimating as inorganic P, which we shall hereinafter refer to as gel P, cannot be inorganic phosphate. Gel P, unlike inorganic P, cannot be removed to any significant extent merely by exhaustive washing of the gel with cold salt solution. Nonetheless gel P estimates as inorganic P in the procedure of Lowry and Lopez (2) which has been devised for the determination of labile phosphate esters, and in other procedures for distinguishing between inorganic phosphate and phosphate esters.

Dynamics of Incorporation Reaction

The following experiment illustrates that radioactive P becomes incorporated almost as quickly as the procedure of transferring the enzyme gel from the manometer cup to the centrifuge tube can be carried out. The oxidation of α-ketoglutarate was carried out in presence of 20 μ-moles of unlabeled phosphate for 15–20m in. at 25°C. At the end of this period 20 μ-moles of radioactive phosphate were quickly introduced into the reaction vessel. The cup contents (within 30 sec.) were then poured into cold salt solution. The residue was washed twice and then tested for radioactivity. The

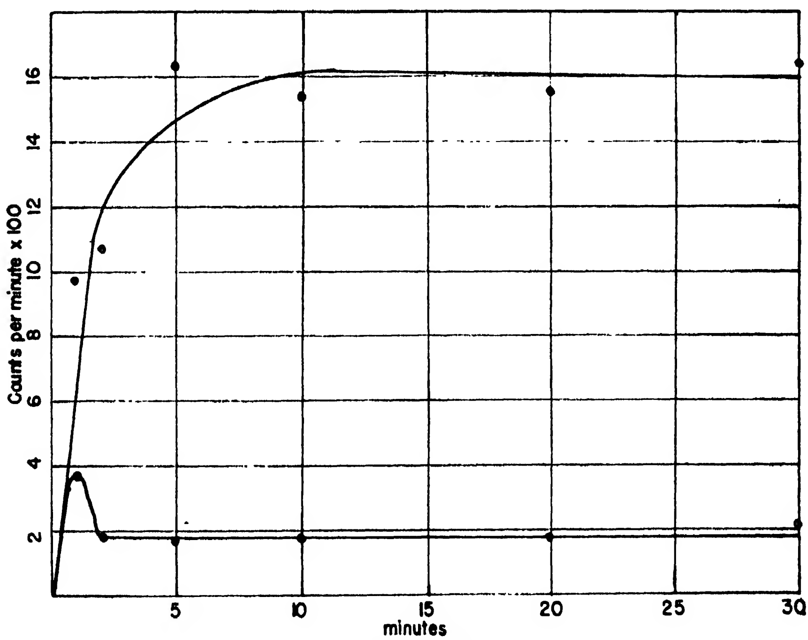

FIG. 1. Radioactivity of cyclophorase gel after incubation with labeled inorganic phosphate for various periods. The experiment was set up in twelve 100-ml. beakers in an air atmosphere. Each beaker contained 0.3 ml. of 0.1 *M* labeled inorganic phosphate, 0.3 ml. of 0.01 *M* AMP (adenosine-5-phosphate), 0.2 ml. of 0.02 *M* $MgSO_4$, 1.0 ml. of a suspension of R_3L (liver cyclophorase), 0.3 ml. of 0.1 *M* α-ketoglutarate as indicated, and water to a final volume of 3.0 ml. Temperature 25°. An identical setup in Warburg vessels with alkali in the center well and air in the gas phase had oxygen uptakes in 10 min. of 0.3 μ-atoms for the blank and 8.0 μ-atoms for the experimental.

blank activity was 89 counts/min. whereas that of the experimental was 898 counts/min.

The incorporation process, although measurable after a fraction of a minute, does not attain its peak before 3–5 min. at 25°C. or some 30 min. at 0°C. Figs. 1 and 2 show incorporation as a function of time at 25°C. This time lag may be explained on the assumption that a certain amount of incorporation accompanies each oxidative step. Over a small time interval the extent of incorporation is determined by a single oxidative process. Over a longer period of time with generation of all the substrates of the citric acid cycle and ancillary metabolites the incorporation approaches the maximum attainable when every oxidative reaction is participating.

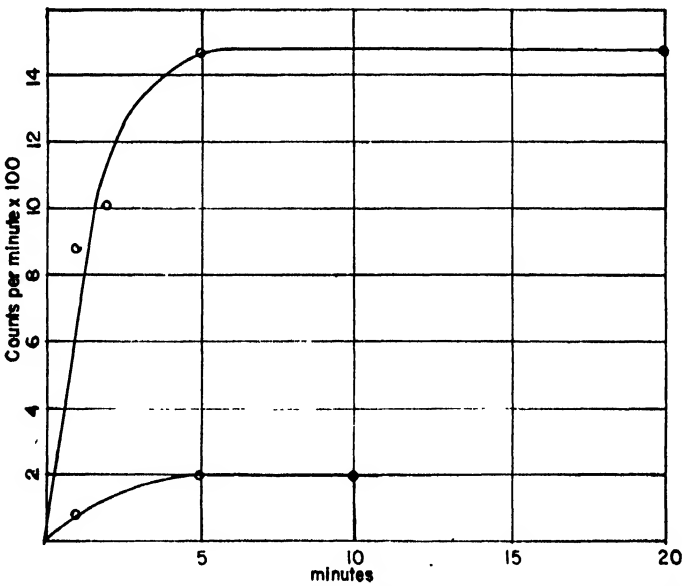

Fig. 2. Uptake of radioactive phosphate after incubation with unlabeled phosphate. Time curve for 25°C. The experimental setup was as described in Table I. The radioactive phosphate was tipped in from the sidearm 15–22 min. after the vessels were placed in the bath. The vessel contents were poured into cold KCl at the indicated time after this tip-in.

If one splits off radioactive P from the experimental gel with acid the radioactivity of the extractable P per μ-mole of P (after acid hydrolysis to convert any pyrophosphate to inorganic phosphate) is identical with the value for the original buffer within the limits of experimental error (*cf.* Table II). In other words there has been complete equilibration between inorganic phosphate in the medium and gel P. In the case of the gel P of the blank the specific activity varies from about 0.5 of the value for the experimental to smaller values depending on the temperature and duration of the experiment. Thus there is only partial equilibration in the absence of added substrate and even this may be attributable largely to oxidation of substrates released by autolysis of the gel.

TABLE II

Specific Radioactivity of Extractable Phosphate in Cyclophorase Gel

Additions	Specific radioactivity (counts/sec./μ-mole inorganic P)			
	Medium[a]		Gel[b]	
	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis
Fluoride	—	430	550	385
No fluoride	430	430	521	441

[a] Supernatant of first wash.

[b] Five per cent trichloroacetic acid extract of thrice washed gel.

Conditions: 1 ml. of R_3K per flask, 38°, oxygen in gas space; 15 min. incubation, α-ketoglutarate as substrate. Other additions and details as in legend of Table I.

It may be appropriate at this point to discuss the experimental evidence bearing on the nature and extent of phosphate incorporation in the blank. As shown in Table III there is more incorporation when radioactive phosphate is present in the blank run from the start of the experiment than when added 15 min. after the start of the experiment. Initially some substrate is present in the blank despite exhaustive washing. With time however the substrate is depleted and the extent of incorporation is correspondingly reduced. The level of gel P provides a convenient index of the extent to which substrate is contributing to the phosphate incorporation in the blank. When some residual oxidation is proceeding in the blank at 25°C. or 38°C. the level of gel P declines only slightly. Once the residual oxidation comes to a halt the level of gel P drops sharply to a base line level and at that point there is a maximum difference between incorporation in the experimental and blank respectively. Since incorporation represents a balance between the synthesis and breakdown of gel P the cessation of synthesis is immediately registered by the level of P^{32} incorporated. At 38°C. the

TABLE III

Uptake of Radioactive Phosphate after Incubation of Cyclophorase Gel with Unlabeled Phosphate

Conditions		Radioactivity of washed gel *counts/min.*
Radioactive phosphate in cup at outset	Blank	650
	Experimental	3080
Radioactive phosphate tipped in after 15 min.	Blank	351
	Blank	355
	Experimental	2240
	Experimental	2100

Conditions: 1 ml. R_3K per flask; oxygen in gas space; 25°C.; 25 min. incubation; α-ketoglutarate as substrate. Other additions and details as in legend for Table I.

TABLE IV

P^{32} Incorporation at 0°C. in Cyclophorase Pretreated with Ferricyanide[a]

	Time of experiment			
	1.5 min.	*2 min.*	*5 min.*	*20 min.*
	Radioactivity of washed gel *counts/min.*			
Blank	191	257	326	318
Experimental	306	404	637	1030

[a] Rabbit kidney cyclophorase at the second residue stage (R_2K) was allowed to stand at 0°C. for 20 min. with 1 μ-mole of ferricyanide/ml. of gel and then washed with 25 volumes of cold 0.9% potassium chloride. Conditions of experiment: 0°C.; 1.5–20 min. duration; α-ketoglutarate as substrate. Other details as in legend for Table I.

When pretreatment of the kidney residue with ferricyanide was omitted the ratio of radioactivity in blank and experimental was essentially 1 after a 20 min. incubation period.

depletion of substrate in the blank is so rapid that after a matter of minutes a maximum experimental over blank incorporation ratio is attained. At 0°C. the depletion of endogenous substrate in the blank is relatively slow and correspondingly the discharge of gel P is not a rapid process. Thus under these conditions the largest differential is obtained when residual substrate is reduced to the lowest possible level before the preparation is used in an experiment. For practical reasons, therefore, we have found it best to treat the enzyme with ferricyanide in order to reduce the activity of the blank to a satisfactory low level (*cf.* Table IV).

There is an important difference between the behavior of the blank at 38°C. and at 0°C. When oxidation in the blank due to residual substrate comes to a halt at 38°C. the cyclophorase gel undergoes an irreversible change. The level of gel P declines to a low value which cannot be raised to the original level merely by adding substrate nor can the activity of the system be regenerated by adding substrate. At 0°C. it is possible to reduce the level of gel P to the base line value and then restore the situation merely by the oxidation of substrate in the presence of phosphate. Thus the process of depleting and regenerating gel P can be repeated at 0°C. since for short experimental periods irreversible processes can be minimized. This aspect of the problem is discussed more fully in an accompanying communication of this series (3).

From these observations it follows that the levels of gel P^{31} and gel P^{32}, although related to the same fundamental process, do not always run parallel. For example at 0°C. a point can be reached where substrate has just been exhausted in the blank but the level of gel P has not yet dropped. Under these conditions a high experimental to blank ratio will be found when P^{32} is measured; but essentially a ratio of one will be found when the level of gel P in the experimental and blank is determined. The lack of substrate will show up at once in experiments which record the extent of equilibration but only more gradually in experiments which record the level of gel P.

Incorporation in Presence of Different Substrates

All the oxidations catalyzed by the rabbit cyclophorase system are accompanied by incorporation of radioactive P (*cf.* Table V). The same results obtain if one merely measures the level of gel P (*cf.* Table VI). There are various substances like L-alanine and fatty acids which are not oxidized as such though their oxidation can be mediated by members of the citric acid cycle. These substances do not lead to incorporation of P^{31} or P^{32}. In presence of α-ketoglutarate or any other member of the citric acid cycle fatty acids and their derivatives increase the extent of P^{32} incorporation well above the level attained with the sparking substance alone.

TABLE V

Phosphate Incorporation in the Presence of Different Substrates

Experiment	Substrate	Oxygen uptake	Radioactivity of washed gels
		μ-atoms	*counts/min.*
1	None	0.1	640
	α-Ketoglutarate	14.4	3390
	Citrate	12.6	2240
	Isocitrate	14.7	2270
	Succinate	11.5	3260
	Fumarate	4.3	3080
	Malate	4.4	3040
2	None	0.6	576
	α-Ketoglutarate	10.7	2400
	Oxaloacetate	5.6	1820
	Pyruvate	2.0	2430
	Proline	5.5	2880
	β-Hydroxybutyrate	5.9	3200
3	None	0	480
	Butyrate	0.3	480
	L-Alanine	0	480
4	None	0.9	1660
	α-Ketoglutarate	7.0	2440
	α-Ketoglutarate + valerate	8.2	3680
	α-Ketoglutarate + α,β-pentenoate	9.0	3360
	α-Ketoglutarate + β-hydroxyvalerate	8.6	3390
	α-Ketoglutarate + β-ketovalerate	8.5	3080
	α-Ketoglutarate + acetate	7.7	2980

Conditions: 1 ml. R_3K per flask; oxygen in gas space; 25°C.; 10 min. incubation; 30 μ-moles of each substrate was used. Other details as in legend for Table I. Initial radioactivity about 150,000 counts/min.

TABLE VI

Gel P Level after Incubation with Various Substrates

Substrates	Oxygen absorbed *μ-atoms*	Gel P *μ-moles*
None	2	0.31
Butyrate	0	0.30
α-Ketoglutarate	32	1.20
Succinate	40	1.22
Fumarate	30	1.25
L-Glutamate	34	1.18
L-Proline	33	1.23

Conditions: 1 ml. R_3K; oxygen in gas space; 38°C.; 15 min. incubation; 30 μ-moles of substrate, 20 μ-moles inorganic phosphate, 4 μ-moles magnesium chloride, and 3 μ-moles adenosine-5-phosphate.

Inhibitors of Phosphate Incorporation

Arsenite and capryl alcohol completely abolish oxidation and oxidative phosphorylation in the cyclophorase system. To a comparable degree they abolish the incorporation phenomenon (*cf.* Table VII). Gramicidin and 2,4-dinitrophenol do not inhibit many of the oxidation steps and only partially inhibit the rest. However, they are highly effective in their capacity to prevent the esterification of inorganic phosphate. It was therefore of great interest to know whether these reagents exerted any effect on P^{32} incorporation. The results showed slight or partial inhibition by these two reagents.

TABLE VII

Suppression of P^{31} and P^{32} Incorporation by Arsenite, Gramicidin, Dinitrophenol, and Capryl Alcohol

	Oxygen absorbed	Inorganic P in washed gel	Radioactivity in washed gel
	μ-atoms	*μ-moles*	*counts/min.*
Blank	2	0.19	90
Experimental	40	1.36	1270
Experimental+0.00033 *M* dinitrophenol	38	0.71	785
Experimental+0.01% gramicidin	43	1.14	1100
Experimental+0.01 *M* arsenite	2	0.27	156
Experimental+1 drop capryl alcohol	7	0.18	60

Conditions: 1.5 ml. R_3K; oxygen in gas space; 38°C.; 15 min. incubation; α-ketoglutarate as substrate. Other details as in Table I. Initial radioactivity per manometer cup about 50,000 counts/min.

Replacement of Oxygen by Ferricyanide

It has been previously shown that ferricyanide can replace oxygen in the reactions of the cyclophorase system and furthermore that esterification of inorganic phosphate accompanies oxidations brought about by ferricyanide. Incorporation of P^{32} is also observed in systems where ferricyanide is used as the oxidizing agent (*cf.* Table VIII). Arsenite, dinitrophenol, and gramicidin all inhibit this incorporation.

TABLE VIII

Incorporation of P^{32} in a System with Ferricyanide as Oxidizing Agent

	CO_2 evolved *µ-moles*	Radioactivity of washed gel *counts/min.*
Blank	0	464
Blank+0.0014 *M* dinitrophenol	0	500
Blank+0.1% gramicidin	0	528
Experimental	12.5	1822
Experimental+0.0014 *M* dinitrophenol	4.6	720
Experimental+0.1% gramicidin	3.5	1022

Conditions: Gas phase 95% nitrogen, 5% carbon dioxide; 25°C.; 15 min. incubation. Other details as in Table I. Initial radioactivity per flask was about 60,000 counts/min.

P^{32} Incorporation in a Phosphate-Deficient Medium

When the cyclophorase gel is brought into contact with radioactive phosphate, the inorganic phosphate level of which is negligible, from 40–60% of the total radioactivity is found in the washed experimental gel and some 5–10% in the washed blank gel (*cf.* Table IX). It is to be noted that the radioactivity of the experimental gel is almost unaffected by washing. These experiments were carried out in the absence of any added phosphate. There was, of course, a measurable amount of gel P already

TABLE IX

P^{32} Incorporation in a Phosphate-Deficient Medium

Fraction	Distribution of radioactivity (per cent of radioactivity originally added to the medium)	
	0.2 ml. P^{32} solution added to medium	0.8 ml. P^{32} solution added to medium
Final washed experimental gel	48	52
Supernatant of first washing	46	40
Supernatant of second washing	6	7
Supernatant of third washing	2	2

Conditions: 1.0 ml. R_1K; 0°C.; 20 min. duration; α-ketoglutarate as substrate; no phosphate was added to the medium other than the negligible amount in the P^{32} solution. Washings were made with 25 volumes of 0.9% potassium chloride at 0°C. In blank preparation (no α-ketoglutarate added) over 90% of the radioactivity was washed out of the gels in the first two washings.

present in the enzyme which can be converted into inorganic phosphate. During active oxidation, available P is partitioned between the gel P on the one hand and the inorganic P in the medium on the other. When there is no added phosphate the gel P is the principal source of inorganic P. At equilibrium some half is in the gel and the rest in the medium. When one adds successively larger amounts of inorganic phosphate in the presence of radioactive marker it can be shown that the radioactivity taken up by the gel corresponds essentially to the same number of μ-moles of P—making due allowance for the fact that the reaction is not complete at low phosphate concentration. In other words the product radioactivity in the gel × total phosphate content of the medium in μ-moles (added P plus initial gel P) does not exceed a variation of 33% over the range studied (*cf.* Table X).

TABLE X

Effect of P^{31} Level on Incorporation of P^{32}

Gel P+inorganic P added to medium μ-moles	Radioactivity of washed gel counts/min.	1×2
0.94	3940	3700
1.44	2820	4060
1.94	2330	4460
2.94	1545	4540
4.94	1040	5140
10.94	515	5630

Conditions: 1 ml. R_3K; 0°C.; 20 min.; α-ketoglutarate as substrate; the initial gel P level was 0.94 μ-mole/ml. R_3K; the radioactivity of the labeled phosphate added to each flask was 8750 counts/min.

The experiment illustrates clearly that gel P is in complete equilibrium with the pool of inorganic P in the medium and that only a definite amount of phosphate can be taken up by the gel essentially independent of the external level of inorganic phosphate above a rather low saturation level (*cf.* Table XI).

TABLE XI

Level of Gel P as a Function of the Phosphate Level in the Medium

Inorganic P added per flask μ-moles	Oxygen absorbed μ-atoms	Gel P in final washed gel
0	8.3	0.30
1	12.4	0.46
2	16.2	0.99
4	19.3	1.19
8	21.4	1.25

Conditions: 1 ml. R_3K; 38°C.; 15 min. incubation; α-ketoglutarate as substrate (30 μ-moles); magnesium chloride (4 μ-moles) and adenosine-5-phosphate (3 μ-moles).

Discharge of Gel P^{32}

When a washed radioactive gel (from an active oxidation run) is allowed to stand at 0°C. there is only a slight decline of bound radioactivity with time. Exposure of such a gel to capryl alcohol, arsenite, or deionized water at 0°C., incubation at 38°C., or freezing and thawing lead to almost complete loss of radioactivity from the gel to the suspension fluid (*cf.* Table XII). Essentially the same results obtain when the level of gel P is measured before and after these treatments.

TABLE XII

Liberation of Incorporated P^{32} from Cyclophorase Gel under Various Conditions

Conditions	Radioactivity of washed gel: Blank (*counts/min.*)	Radioactivity of washed gel: Experimental
Experiment I		
Washed twice with 0.9% potassium chloride at 0°C.	280	867
Frozen and thawed 4 times before second wash	128	172
Treated with 5% trichloroacetic acid at 0°C. before second wash	168	195
Experiment II		
Washed twice with 0.9% potassium chloride at 0°C.	547	1610
Washed twice with deionized water at 0°C.	167	235
Experiment III		
Washed twice with 0.9% potassium chloride at 0°C.	302	637
Washed twice with 0.9% potassium chloride containing a drop of capryl alcohol at 0°C.	122	245
Incubated 10 min. at 38°C. before second wash	130	150
Incubated 2 min. at 38°C. with 0.01 *M* arsenite before washing	105	120

General procedure as in Table I; R_3K was used.

Presence of Hydrolyzable P in the Gel

A small portion of the acid-extractable phosphate of the gel is present in the form of a pyrophosphate but this moiety is not hydrolyzed during the trichloroacetic acid extraction. In general the experimental gel contains more pyrophosphate than the blank gel and the presence of fluoride promotes the accumulation of hydrolyzable phosphate (*cf.* Table XIII). In paper XIII of this series (4), Dr. Albaum deals with the isolation of radioactive adenosine polyphosphate from the radioactive gel. As yet insufficient data are at hand to state under what conditions or the extent to which gel

TABLE XIII

Hydrolyzable P in Cyclophorase after Incubation with and without Substrate

	Oxygen absorbed	Inorganic P in washed gel	Hydrolyzable P in washed gel
	μ-atoms	*μ-moles*	*μ-moles*
Blank	2	0.40	0.14
Experimental	52	1.22	0.52

Conditions: 1.5 ml. R_1K; 38°C.; 15-min. incubation; α-ketoglutarate as substrate; 0.033 *M* sodium fluoride and usual amounts of magnesium chloride and adenylic acid. Hydrolysis was carried out by heating the trichloroacetic acid extract of the washed gel for 7 min. at 100°C. in 1.0 *N* H_2SO_4.

P can be converted into phosphate that estimates as hydrolyzable P rather than as inorganic P. In any event the labile P of the hydrolyzable moiety of gel P is in complete equilibrium with the inorganic P of the medium as shown by the experiments of Albaum.

Incorporation of P^{32} and Oxidative Phosphorylation

There is evidence from a variety of experiments that the ability to incorporate P^{32} and the ability to carry on oxidative phosphorylation always run parallel. Thus the succinic oxidase in the intact cyclophorase unit (in the mitochondrial bodies) can carry out both processes. When the mitochondrial bodies are fragmented by shearing forces the succinic oxidase of such a disrupted unit is found to be unable to carry on either of these two functions. Other oxidases like the malic and the α-ketoglutaric enzymes show a similar behavior.

TABLE XIV

Phosphate Esterification by the Cyclophorase System after Preheating the Enzyme with and without Fluoride

	Oxygen absorbed	Inogranic P esterified
	μ-atoms	%
Fresh cyclophorase	55	98
Cyclophorase preheated 10 min. at 38°C.	48	34
Cyclophorase preheated 10 min. with 0.033 *M* fluoride	43	76

Conditions: 1.0 ml. R_1K; 38°C.; oxygen in gas space; 15-min. incubation with substrate; 40 μ-moles inorganic phosphate, 0.1 molar fructose and 0.05 ml. of a yeast hexokinase preparation were added to each flask in addition to magnesium chloride and adenylic acid. Succinate was the substrate. The preheated vessels were shaken 10 min. at 38°C. before 0.1 ml. of molar succinate was tipped in. In the case of the mixture heated without fluoride, fluoride was tipped in after the preheating period to make its concentration 0.033 *M*; this level of fluoride was thus present in all flasks during incubation with substrate.

There may be technical difficulties which make it difficult to demonstrate oxidative phosphorylation in a system containing disrupted mitochondrial particles; consequently we cannot exclude the possibility that under proper conditions the power of oxidative phosphorylation can be conserved even when the mitochondrial unit is no longer intact.

TABLE XV

P^{32} Incorporation by Cyclophorase Gel after Preheating with and without Fluoride

	Oxygen uptake *μ-atoms*	Radioactivity in washed gel *counts/min.*
Blank, preheated 10 min. at 38°C.	2	512
Blank, preheated with 0.022 *M* fluoride	2	680
Experimental, preheated	4	690
Experimental, preheated with 0.022 *M* fluoride	10	2560

Conditions: The substrate (α-ketoglutarate) was tipped into the experimental vessels after the preheating period. At the same time fluoride was tipped into the mixtures which had not contained fluoride to make its concentration 0.022 *M*. 1.0 ml. R_3K was used. Other conditions as in Table I.

Exposure of the cyclophorase gel to 38°C. rapidly leads to the loss of the ability of the succinic oxidase to carry on either oxidative phosphorylation or the incorporation of P^{32}. Over short exposure periods (*e.g.*, 10 min.) fluoride protects the gel against the total loss of these two functions (*cf.* Tables XIV and XV).

P^{32} Incorporation in a Mitochondrial Preparation

Elsewhere Dr. John Harman will present evidence that the mitochondria are the active constituents of our standard liver and kidney cyclophorase preparations. Using the procedure of Schneider *et al.* (5) for the preparation of mitochondria which are essentially free of all interfering structural elements it could readily be demonstrated that such a preparation is capable of both P^{32} incorporation and oxidative phosphorylation (*cf.* Table XVI).

TABLE XVI

P^{32} Incorporation in a Mitochondrial Preparation

	Oxygen absorbed *μ-moles*	Radioactivity *counts/min.*
Blank at 0°C.	—	118
Experimental at 0°C.	—	854
Blank at 38°C.	0	101
Experimental at 38°C.	7.2	768

Conditions as in Table I except that a kidney mitochondrial preparation was used instead of R_3K. The preparation was made according to Schneider (5) after homogenizing the tissue in a Waring blendor.

Incorporation of Other Radioactive Elements

The incorporation of P^{32} has been referred to a chemical reaction between inorganic phosphate and some coenzyme in the cyclophorase gel. Some light on the specificity of this incorporation is shed by experiments in which other radioactive elements were tested, *viz.*, Na^{23} (NaCl), K^{42} (KCl), and C^{14} (CH_3COONa). In none of these experiments was there any evidence that the experimental gel was more radioactive than the blank nor was there any indication that any of these radioactive ions could be retained by the gel through successive washings.

Discussion

The experimental data presented in this communication permit the following conclusions: (*a*) coincident with any oxidation process in the intact cyclophorase system inorganic phosphate becomes esterified or incorporated in the enzyme gel in a highly labile form which for convenience may be referred to as gel P; (*b*) under conditions of active oxidation gel P equilibrates rapidly with inorganic P in the medium; (*c*) any reagent which abolishes oxidation also abolishes incorporation of P^{32} and leads to the discharge of gel P initially present in the enzyme; (*d*) gel P, although estimating as inorganic P, is not inorganic P; (*e*) the maximum level of gel P attainable (about 1 μ-mole/50 mg. dry weight of gel) is fairly constant from one preparation to another.

The main outstanding problems now are (*a*) the nature of gel P and (*b*) the nature of the components in the cyclophorase complex to which the labile P is attached. As yet there is no clear evidence available as to the type of phosphoric ester by which gel P (other than the pyrophosphate moiety) may be represented. It has none of the properties of an acyl phosphate nor does it resemble any known labile phosphoric ester. Our present hypothesis is that phosphate is taken up coincident with the reduction (and perhaps oxidation) of the pyridine nucleotides and is incorporated in the form of a labile phosphoric ester which estimates as inorganic phosphate under the acid conditions of the Fiske-Subbarow or Lowry-Lopez methods of estimation.

Recently Hummel and Lindberg (6) have reported that P^{32} was present in flavinadenine dinucleotide isolated by relatively drastic procedures from homogenates undergoing oxidative phosphorylation in presence of radioactive phosphate. In the light of our experience on the extreme instability of gel P there is no possibility that the stable marked phosphate group in the flavindinucleotide fraction which Hummel and Lindberg have separated could be regarded as existing

in the same form as the bulk of the incorporated P^{32} in the cyclophorase gel.

EXPERIMENTAL II

Preparation of the Enzyme

Both rabbit liver and kidney cyclophorase suspensions at the stage of the third residue were prepared according to the method described in detail by Green *et al.* (7).

Washing Procedure

Various procedures have been used but mention will be made here of only the most acceptable and convenient procedure. The contents of a single manometer vessel were washed into about 50 ml. of cold 0.9% potassium chloride solution. The suspension, after being stirred briefly with a mechanical stirrer, was centrifuged at 0°C. Potassium chloride was then added to about 50 ml. and after thorough mixing of the suspension the residue was again removed by centrifugation. All manupilations must be carried out to 0°C. and expeditiously to minimize loss of radioactivity in the experimental run.

Measurement of Radioactivity

The washed residues were dissolved in 5 ml. of 50% potassium hydroxide. One ml. of the solution was pipetted into a shallow tin pan of uniform dimensions and the pan was placed in a Geiger-Mueller counter for measurement of radioactivity.

Phosphate Estimation

Cf. Cross *et al.* (1) for full details.

SUMMARY

1. During the oxidation of any substrate of the cyclophorase system in the presence of radioactive phosphate, P^{32} becomes incorporated within the gel.

2. Under the conditions for determining inorganic phosphate by the Fiske-Subbarow or Lowry-Lopez methods, gel P estimates as inorganic P.

3. Reagents like arsenite or capryl alcohol which paralyze oxidation also abolish P^{32} incorporation. Gramicidin and dinitrophenol in the minimal concentrations necessary for blocking esterification of glucose in presence of the combined cyclophorase-hexokinase system only partially inhibit incorporation of P^{32}.

4. Gel P is readily discharged by any of the reagents which are known to transform the oxidases of the cyclophorase system into their classical counterparts.

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Studies on the Cyclophorase System. XIII. Distribution of Radioactivity in Various Phosphorus-Containing Compounds of the Cyclophorase Gel

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Introduction

In a previous communication of this series (1) the incorporation of P^{32} into the cyclophorase gel was shown to accompany oxidative activity. The present communication documents the extent of P^{32} incorporation in the various phosphorus esters present in the cyclophorase gel.

The trichloroacetic acid (TCA) filtrates of washed cyclophorase gels from large scale experiments with radioactive phosphate were fractionated first with barium and then with mercury according to the procedure shown in Fig. 1; each fraction was analyzed for radioactivity. Whenever possible each fraction was also analyzed for total phosphorus. (In a few cases aliquots remaining after removal of samples for counts were not sufficiently large to determine total phosphorus with any degree of accuracy.) The results of the analyses are shown in Table I. The experimental run contained α-ketoglutarate as substrate, whereas the blank carried out on exactly the same scale contained arsenite in addition to α-ketoglutarate. The recoveries in terms of radioactivity for the different fractions are shown in the second column. Several interesting points are discernible. The ratio of the radioactivity in the TCA filtrates of experimental and blank is 9.1 to 1. This radioactivity appears to be distributed throughout all the fractions although largely concentrated in fractions 5 and 6. The inorganic phosphate content of the TCA filtrate of the experimental run is 9.1 times as much as is present in the filtrate of the blank run, and as a rough approximation accounts for most of the radioactivity present. Considering the thoroughness of

Washed enzyme treated with 0.25 vol. iced 50% trichloroacetic acid, centrifuged; extraction repeated twice with equal vol. of 10% TCA.

- Residue (1) taken up in 20% KOH.
- Combined supernatants (2). pH adjusted to 8.2, made to volume; treated with 4 vols. ethanol and 0.1 vol. 20% barium acetate; chilled, centrifuged.
  - Barium- and alcohol-insoluble fraction; suspended in H_2O; glacial acetic acid added to pH 2.0; centrifuged.
    - Barium-insoluble, acetic acid-insoluble (6); suspend in H_2O, shaken with Na_2SO_4 and 0.2% acetic acid.
      - Residue (7) taken up in 20% KOH.
      - Supernatant (8) barium-insoluble acetic acid-insoluble $SO_4^=$ -soluble.
    - Barium- and alcohol-insoluble, acetic acid-soluble fraction; treated with 4 vols. ethanol; centrifuged.
      - Residue dissolved in 0.2% acetic acid, pH adjusted to 8.2 with NaOH, centrifuged.
        - Barium-insoluble fraction (5) taken up in 0.2% acetic acid; treated with 0.05 vol. 20% mercuric acetate.
          - Barium-insoluble Mercury-insoluble (12)
          - Barium-insoluble Mercury-soluble (11)
        - Barium-soluble fraction (4) made 0.2% with respect to acetic acid; treated with 0.05 vol. 20% mercuric acetate.
          - Barium-soluble Mercury-insoluble (10)
          - Barium-soluble Mercury-soluble (9)
      - Supernatant. Discarded.
  - Barium-soluble, alcohol-soluble fraction (3); evaporated down under reduced pressure before assay.

FIG. 1. Flowsheet for fractionation of trichloroacetic acid extracts

the preliminary washing procedure prior to treatment with TCA it is improbable that free inorganic phosphate other than that which is incorporated in the gel was present in any considerable amount in the experimental run.

When fraction 5 is treated with mercury the adenine nucleotides

TABLE I

Radioactivity and Phosphate Determinations on Fractions of Trichloroacetic Acid Extracts

Fraction	Blank						Experimental					
	Counts /min. ×100	Per cent of previous fraction	Total P	Inorganic P	μM PO_4 taken up[a]	Per cent equilibration[b]	Counts /min. ×100	Per cent of previous fraction	Total P	Inorganic P	μM PO_4 taken up[a]	Per cent equilibration[b]
			μ-M	μ-M					μ-M	μ-M		
1. TCA residue	40	14	652	—	2.9	0.4	134	5.5	676	—	9.6	1.4
2. TCA supernatant	255	86	50.7	14.1	18.3	36	2310	94.5	208	127	165	79
Total 1 and 2	295	100	703		21.2	3.0	2440	100	884		174	20
		% of 2						*% of 2*				
3. Barium-soluble, alcohol-soluble	0.6	0.23	22	—	0.04	0.20	18	0.78	30	—	0.96	4
4. Barium-soluble	4.7	1.84	6.6	—	0.33	5.0	19.3	0.84	12.5	—	1.4	11.0
5. Barium-insoluble	96.8	38	14.9	10.4	6.9	46	1580	68.4	118	86	113	96
6. Barium-insoluble, acid-insoluble	157	61.5	—	—	11.2	—	568	24.6	—	—	40	—
Total 3, 4, 5, and 6	259	102	—	—	18.5	—	2190	94.6	—	—	155	—

TABLE I—*Continued*

Fraction	Blank						Experimental					
	Counts /min. ×100	Per cent of previous fraction	Total P	Inorganic P	μM PO_4 taken up[a]	Per cent equilibration[a]	Counts /min. ×100	Per cent of previous fraction	Total P	Inorganic P	μM PO_4 taken up[a]	Per cent equilibration[b]
		% of 4+5	*μ-M*	*μ-M*				*% of 4+5*	*μ-M*	*μ-M*		
9. Barium-soluble, mercury-soluble	1.86	1.84	2.1	1.1	1.29	6.3	13.5	0.84	5.8	1.6	0.96	16.5
10. Barium-soluble, mercury-insoluble	1.21	1.19	3.0	0.8	0.09	2.9	3.7	0.23	4.8	1.2	0.25	5.4
11. Barium-insoluble, mercury-soluble	75.8	74.8	11.9	9.6	5.4	46	826	51.8	59	56.5	59	100
12. Barium-insoluble, mercury-insoluble	12.7	12.5	4.6	0.5	0.90	20	510	31.9	50	30	36	72
Total 9, 10, 11, 12.	91.5	90	21.6	12	6.6	30	1350	84.8	120	89.3	97	81

[a] This column indicates the amount of phosphate derived from the surrounding medium during the incubation period. The figures are obtained by dividing the "counts/min." by the specific activity of the original medium.

[b] This column indicates the percentage of the "Total P" which has equilibrated with the radioactive P in the original medium; the figures are derived by dividing "μ-moles P taken up" by "Total P."

The experimental setup contained 100 ml. of liver cyclophorase at the third residue stage (R_3L), 0.01 *M* $PO_4^{=}$ buffer at pH 7.3, 0.0066 *M* $MgCl_2$, 0.001 *M* adenosine-5-phosphate, and 0.005 *M* α-ketoglutarate; 0.9% potassium chloride was added to a total volume of 300 ml. The blank did not contain α-ketoglutarate and 0.01 *M* arsenite was added. The mixtures were held at 25°C. for 15 min. with O_2 bubbled through and were then washed 3 times with 20 volumes of 0.9% potassium chloride at 0°C. The washed gels were precipitated and washed with cold 10% trichloroacetic acid and the extract was fractionated according to Fig. 1. Radioactivity measurements were made in a Geiger-Muller counter on samples suspended in 8% potassium hydroxide. A correction was made for a background count of 25 counts/min. A small aliquot of the enzyme was checked for oxidative activity in manometer cups.

form insoluble salts, while the less insoluble inorganic phosphate salts and other barium-insoluble esters stay in the supernatant solution. This type of fractionation is shown in Table I, fractions 11 and 12. In this case the fractionation was not clear cut. In a repeat experiment shown in Table II fraction 5 was treated in the same way; here the fractionation was more satisfactory. The barium-insoluble, mercury-supernatant contains 96% of the inorganic P present in fraction 5. The total inorganic P of fractions 11 and 12 is greater than that present in fraction 5, suggesting that some breakdown may have occurred during the fractionation.

Fraction 12, shown in Table II, normally expected to contain adenine polyphosphates, was freed of barium and assayed for adenine nucleotides enzymatically in the spectrophotometer. No deamination occurred in the presence of a rabbit deaminase alone, using the method of Kalckar (2)—a result which indicates the absence of free adenylic acid. When the reaction was carried out, however, in the presence of glucose, magnesium chloride, myokinase, hexokinase, and the deaminase, a decrease in density at 265 mμ was observed (from 0.326 to 0.064 in 15 min.) indicating the presence of either adenosine diphosphate or adenosine triphosphate. If a portion of the radioactivity in this fraction is indeed in the form of adenosine polyphosphate, then refractionation of the material after the action of myokinase and hexokinase should result in a shift in distribution of the phosphorus as well as in the radioactivity. Since glucose-6-phosphate would be expected to be found in the barium-soluble, mercury-soluble fraction, and increase in the total P and radioactivity of this fraction, along with a decrease in the barium-insoluble, mercury-insoluble fraction, would indicate that the radioactivity initially present in the original fraction 5 was present as adenosine polyphosphate. The data shown at the bottom of Table II bear this out.

Fraction 6 is of special interest. It contains considerable radioactivity. It is material insoluble in 0.2% acetic acid and also barium-insoluble, and therefore should not contain ADP or ATP as ordinarily encountered. It can be brought into solution partially by suspending in acidified Na_2SO_4 with stirring and in this way exchanging the barium for sodium. This solution, fraction 8 (Table II), contains most of the radioactivity and phosphorus of the fraction. When examined enzymatically in the spectrophotometer, it contains no free adenylic acid, but has considerable quantities of adenosine polyphosphate as shown by the spectro-

TABLE II

Radioactivity and Phosphate Determinations on Fractions of a Trichloroacetic Acid Extract

Fraction	Counts/min. ×1000	Per cent of previous fraction	Total P	Inorganic P	7-min. Labile P	μ-M PO_4 taken up	Per cent equilibration	Adenine
			μ-M	μ-M				μ-M
3. Barium-soluble, alcohol-soluble	67	1.4	35.5	—	—	4.7	13.2	
4. Barium-soluble	665	13.7	72.1	30.1	8.1	47	64	14.6
5. Barium-insoluble	3450	70.8	297	223	29.3	242	81	23.5
7. Acid-insoluble, $SO_4^{=}$-insoluble	160	3.3	—	—	—	11.2	—	—
8. Acid-insoluble, $SO_4^{=}$-soluble	442	9.1	41.5	16.7	8.5	31	76	8.2
Total (3–8)	4870	100	446+	270+	46+	342	77	
		% of 4+5						
9. Barium-soluble, mercury-soluble	647	15.7	51	37.6		46	90	
10. Barium-soluble, mercury-insoluble	13.4	0.3	4.8	1.7		1.0	20	3.4
11. Barium-insoluble, mercury-soluble	2920	70.9	216	214		206	96	
12. Barium-insoluble, mercury-insoluble	425	10.3	61.1	16.2		30	49	19.9
Total (9–12)	4010	97.2	333	269		282	84	
Fraction 12 after enzyme treatment[a]								
16. Barium-soluble, mercury-soluble	326	76.7	30.7	12.7		23	74	
17. Barium-soluble, mercury-insoluble	6.4	1.5	4.3	0		0.4	10.6	
18. Barium-insoluble, mercury-soluble	65	15.3	8.1	5.2		4.5	57	
19. Barium-insoluble, mercury-insoluble	3.2	0.8	2.0	2.0		0.3	84	
Total (16–19)	401	94.3	45.1	19.9		28	63	

[a] Fraction 12 was treated with myokinase and hexokinase as described in the text before separation into fractions 16–19.

photometric test referred to above. The nature of this material is still not clear.

The insoluble material following treatment with TCA contains the bulk of the phosphorus originally present in the cyclophorase gel. Analysis has shown the residue to contain nucleic acid in considerable amounts. Apparently, as Friedkin and Lehninger (3) have found, there is little incorporation of radioactive phosphate into the nucleic acid moiety of the cyclophorase complex.

By comparing the radioactivity per unit of phosphorus in the original medium with corresponding values for the various fractions, it was found that there was practically complete equilibration in the experimental gel fractions 2, 5, 11, and 12, with relatively little equilibration in the other fractions. In each case the extent of equilibration in the blank gel fractions was about one-half that in the corresponding experimental gel fractions.

Experimental

The details of the method of preparation of the liver cyclophorase and the measurement of radioactivity are provided in previous communications (1,4).

Analytical Procedures

Phosphorus was determined by the method of Fiske and Subbarow (5); digestion for total phosphorus was carried out in 10 N H_2SO_4; labile phosphorus was determined after hydrolysis in 1 N HCl at 100°C. for 7 min. Adenylic acid was assayed spectrophotometrically using a rabbit deaminase according to the method of Kalckar (2). Adenine polyphosphate was assayed by combining the deaminase-myokinase method of Kalckar with a hexokinase system (6). Ribose was determined by the method of Mejbaum (7) using ribose as a standard and a 45-min. heating period. Nucleic acids were measured after fractionation according to the procedure of Schneider (8).

Summary

A study was made of the distribution of P^{32} in various phosphorus-containing compounds extracted with trichloroacetic acid from a liver cyclophorase gel which had been allowed to carry on active oxidation in presence of radioactive phosphate and which was finally washed free of all soluble material. The bulk of the incorporated P^{32} was found in the trichloroacetic acid extract in a form estimating as inorganic phosphate. The adenine polyphosphate fraction was also highly radioactive and the radioactivity resided in the pyrophosphate moiety. After treatment of the adenine polyphosphate with glucose in presence of

hexokinase and myokinase, the radioactivity was transferred to the glucose phosphate fraction. Another adenine polyphosphate fraction, not identical with ADP or ATP, was found in the trichloroacetic acid filtrate and this unknown compound was also highly radioactive.

Acknowledgments

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Studies on the Cyclophorase System. XIV. Mechanism of Action of 2,4-Dinitrophenol

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Introduction

In a previous communication by Cross *et al.* (1), it was shown that 2,4-dinitrophenol (DNP) paralyzes the reactions by which inorganic phosphate becomes esterified coincident with oxidation in the cyclophorase system but yet does not inhibit to a comparable degree some of the oxidative steps such as the oxidation of α-ketoglutarate to succinate. In addition, the sparking of fatty acid oxidation by members of the citric acid cycle was found to be unusually sensitive to the inhibitory action of DNP.

In a preliminary note Loomis and Lipmann (2) have made some observations which bear on the mechanism of action of DNP. They observed that in the presence of DNP and in the absence of added inorganic phosphate, the oxidation of glutamate by washed kidney particles proceeded almost maximally whereas in the absence of the reagent a clear phosphate requirement was demonstrable. This has been confirmed in our laboratory. From this result the authors concluded that DNP can replace inorganic phosphate and that oxidation and phosphorylation are independent processes which can be dissociated by appropriate means.

This interpretation was out of line with our experiments which had led us to the view that oxidative phosphorylation in the intact cyclophorase system is an obligatory process in the sense that the esterification of inorganic phosphate cannot be dissociated from the oxido-reduction process. An observation which was made on the action of DNP in discharging gel P (3) suggested another interpretation of the Loomis-Lipmann effect and this communication in essence deals with the extension of this basic observation.

Plan of Experiments

In a previous communication (3) evidence had been presented that when substrates are undergoing oxidation in the cyclophorase system, inorganic phosphate is taken up by the gel in the form of an ester (or esters) of extreme instability. This ester has been referred to previously as gel P, and until there is more precise knowledge of its constitution such designation will be retained. In a cyclophorase system carrying on active oxidation a dynamic equilibrium exists which may be represented as follows:

$$\text{inorganic P} \underset{\text{breakdown}}{\overset{\text{oxidation}}{\rightleftharpoons}} \text{gel P}$$

DNP has the effect of discharging gel P so that the dynamic equilibrium is shifted towards the side of inorganic P. From these observations previously made in studies with the use of P^{32}, the following experimental hypothesis may be formulated. In a cyclophorase gel to which no phosphate has been added there is always in the gel P a potential reservoir of inorganic phosphate which cannot be removed merely by exhaustive washing of the gel. In the absence of added inorganic phosphate the cyclophorase system is not able to function maximally since the leak of inorganic phosphate from the gel is too small to maintain a saturation level in the medium. Such a gel will therefore catalyze a given oxidation more rapidly when supplemented with inorganic phosphate. When DNP is present some of the gel P is discharged and made available in the form of inorganic phosphate and at a level which is adequate to permit the oxidase to function at a higher though usually not maximum, velocity. According to this view DNP does not "replace" inorganic phosphate but merely makes available a form of phosphate which cannot be drawn upon for an adequate supply in the absence of reagent. If such be the case it should follow that a gel pretreated with DNP and then thoroughly washed should not only contain very little gel P but also should show a phosphate requirement whether or not DNP is present.

Experimental

The cyclophorase gels were prepared according to the method of Green *et al.* (4), except that the period of homogenization was shortened to one minute. The procedure for pretreatment is described in Table I. Inorganic phosphate was determined by the method of Fiske and Subbarow (5). For further details of procedure *cf.* Green *et al.* (3).

Results

At 0° and in the presence of $1\text{–}3 \times 10^{-4}$ M DNP from 75–90% of the gel P can be discharged within 30 min. At higher temperatures or concentrations of DNP the discharge of gel P is accompanied by an irreversible inactivation or damaging of the catalytic power of the cyclophorase gel. After treatment, the gel is thoroughly washed with many volumes of cold 0.9% potassium chloride and then tested.

Table I contains the results of a typical experiment. Pretreated kidney gel at the third residue stage was found to contain initially 0.09 μ-moles of gel P/ml. The control R_3K which was treated in exactly the same way (except for exposure to DNP) contained 0.55 μ-moles of gel P/ml. The pretreated kidney gel no longer shows a response to DNP. That is to say, DNP does not replace inorganic phosphate. In contrast to inorganic phosphate, which increases the base line rate 2.3-fold, DNP has no effect. As a consequence of active oxidation in the presence of inorganic phosphate, the level of gel P in the pretreated enzyme rose from 0.09 μ-moles to 0.49 μ-moles P. The slight rise in the level in the

TABLE I

Splitting of Gel P with Dinitrophenol and Reincorporation of Gel P

Enzyme	Additions to medium	Oxygen absorbed	Final gel P	"Initial" gel P
		μ-atoms	*μ-moles*	*μ-moles*
R_2K	20 μ-moles inorganic P	97	1.09	—
R_3K	20 μ-moles inorganic P	65	0.98	0.55
Pretreated R_3K	None	32	0.15	0.09
Pretreated R_3K	20 μ-moles inorganic P	74	0.49	—
Pretreated R_3K	3.3×10^{-5} M DNP	33	0.04	

The R_2K (rabbit kidney cyclophorase at the second residue stage) was treated for 30 min. at 0°C. with 2×10^{-4} M DNP and washed with 50 volumes of 0.9% potassium chloride at 0°C. Each cup contained 0.02 M magnesium chloride, 0.0001 M adenosine-5-phosphate, 0.01 M α-ketoglutarate, 1 ml. of enzyme gel, alkali in the center well and 0.9% potassium chloride to make a final volume of 3.0 ml. The vessels were flushed with oxygen and equilibrated in the bath for 5 min. The experiments were carried out for 30 min. at 38°C. At the end of the experiments the vessels were chilled and the gels were washed twice with 25 volumes of 0.9% potassium chloride at 0°C. Gel P was determined by measuring inorganic phosphate in a 5% trichloroacetic acid extract of the washed gel. The "initial gel P" values were obtained on gels that were kept at 0°C. during the course of the experiment and then washed along with the other gels.

experiment where no phosphate was added is attributable to the inorganic phosphate arising from the breakdown of adenylic acid and phosphoric esters in the gel. Exposed to DNP for a second time the pretreated gel suffers a further decline in gel P.

It has been found that provided the gel is processed carefully the amount of gel P per unit of dry weight[1] is approximately the same in the fresh and "reworked" gels and is much lower in the DNP-treated gel.

The results shown in Tables I to III point up a fundamental difference in the response of a DNP-treated enzyme and an untreated enzyme to DNP. The untreated enzyme works more rapidly in the presence of DNP in a phosphate-deficient medium whereas the velocity of the pretreated enzyme is either unaffected or only slightly stimulated.

TABLE II

Treatment of Liver Cyclophorase with Dinitrophenol and its Subsequent Response to Inorganic Phosphate and Dinitrophenol

Enzyme used	Oxygen absorbed (μ-atoms)		
	No additions to medium	4 μ-moles inorganic phosphate added	DNP added 1×10^{-4} M
R_4L	11.8	24.0	16.3
Pretreated R_4L	14.1	26.3	13.6

Conditions as in Table I except that the experiment was carried out for 15 min. and 0.005 M sodium fluoride was added to the medium. R_2L was washed twice after pretreatment with DNP.

Regardless whether the enzyme has been pretreated or not (provided the treatment has not been too severe) a pronounced response to inorganic phosphate is observed. It should also be pointed out that even in the presence of DNP (to which there is little or no response) the pretreated enzyme is markedly stimulated by inorganic phosphate.

Similar results have been obtained when other substrates of the cyclophorase system (succinate excepted) have been used instead of α-ketoglutarate (*cf.* Table IV).

There is one anomaly that should be mentioned, *viz.* that the rate of

[1] The cyclophorase preparation used in these studies contained approximately 5.0% solids.

TABLE III

Response of Treated Kidney Cyclophorase to Inorganic Phosphate and Dinitrophenol in a Phosphate-Deficient System

Additions to medium	Oxygen absorbed μ-atoms
None	8.8
3.3×10^{-5} *M* DNP	11.6
0.5 μ-moles inorganic P	13.4
2.0 μ-moles inorganic P	26.0
20.0 μ-moles inorganic P	32.1

Conditions: 1.0 ml. pretreated R_4K; 0.01 *M* sodium fluoride; oxygen in gas space; 38°C.; 20 min. incubation.

TABLE IV

Response of Pretreated Kidney Cyclophorase to Inorganic Phosphate and DNP with Various Substrates

Substrate	Oxygen absorbed (μ-atoms)		
	No additions to medium	4 μ-moles inorganic phosphate added	1×10^{-4} DNP added
α-Ketoglutarate	3.9	10.0	2.7
Citrate	4.7	14.4	3.4
Succinate	8.7	10.9	8.8
L-Glutamate	4.3	10.0	2.4

Conditions: 1.0 ml. pretreated R_4K; 0.005 *M* fluoride; oxygen in gas space; 38°C.; 15 min. duration.

oxidation catalyzed by a pretreated gel containing a very low level of gel P is still some 25% of the maximum. In relation to the available P that rate would appear to be excessive unless there has been a partial alteration of the cyclophorase enzymes.

Discharge of Gel P during Aging

On standing at 0°C. for 24–48 hr. the gel P level of kidney cyclophorase gel declines toward the low levels brought about by pretreatment of the gel with DNP. The level in liver cyclophorase declines more rapidly. Table V shows that an aged gel shows a relatively small DNP effect and that DNP cannot serve in the stead of inorganic phosphate.

TABLE V

Response of Aged Kidney Cyclophorase to Inorganic Phosphate and Dinitrophenol

Additions to medium	Oxygen absorbed *µ-atoms*
None	8.7
10 µ-moles inorganic phosphate	18.4
1×10^{-4} *M* DNP	12.4
10 µ-moles inorganic phosphate$+1\times10^{-4}$ *M* DNP	17.2

Conditions: R_2K was aged for 24 hrs. at 0°C. and washed with 50 volumes of cold 0.9% potassium chloride. One ml. of the enzyme gel was used. Oxygen in gas space; 38°C.; 15 min. duration; 0.005 *M* sodium fluoride.

Summary

Dinitrophenol has been shown to be a reagent which contrary to the claims of Loomis and Lipmann does not uncouple oxidative phosphorylation or "replace" inorganic phosphate but rather makes available as inorganic phosphate the phosphate of one or more labile esters which are always present in a freshly prepared enzyme gel. In terms of this discharge phenomenon the observed effects of this reagent can be explained.

Acknowledgment

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The Properties of the Enzyme-Substrate Compounds of Horseradish Peroxidase and Peroxides. III. The Reaction of Complex II with Ascorbic Acid [1]

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Introduction

A very rapid and sensitive method has been developed for studies of the reaction velocity of the enzyme-substrate complex of peroxidase and H_2O_2 with acceptors (reducing substrates) (1). In this method, the utilization of substrate by the enzyme system is measured from the spectrophotometric trace of the concentration of the enzyme-substrate complex as a function of time; the enzyme-substrate complex is used as a spectrophotometric indicator of the substrate concentration. This method is extremely sensitive (the disappearance of less than 1×10^{-6} M peroxide can be measured with an error of less than 5%) and has a general applicability in studies of enzymatic activity in the presence of a small substrate concentration where enzyme inactivation is minimized. Also, the enzymatic activity is satisfactorily measured even though no colored reaction products are measured. And most important the direct measurements of the concentration of the enzyme-substrate complex permit a normalization of all activity data on the basis of a saturated enzyme-substrate complex by a simple formula (see below). Since this method is used extensively in studies of the acceptor specificity of peroxidase, proof of its validity is given here over a much wider range of conditions than was heretofore possible. Such proof depends upon correlations between the overall reaction (the disappearance of substrate) and the kinetics of the enzyme-substrate compound.

[1] This is No. 9 of a series on catalases and peroxidases.

[2] John Simon Guggenheim Memorial Fellow (1946–1948).

The reaction kinetics of horseradish peroxidase and peroxides have been studied spectrophotometrically and have been found to involve two forms of enzyme-substrate complex (2). However, in the presence of an acceptor (reducing substrate) such as ascorbic acid, the transition from the primary (I) to the secondary (II) peroxidase-peroxide complex is very rapid, in fact, the reaction of Complex II with the acceptor is usually the rate-determining step in this enzymatic action (3). Thus, Complex II is the Michaelis enzyme-substrate complex that governs the activity of peroxidase.

A detailed demonstration of the applicability of the theory of Michaelis and Menten to the kinetics of horseradish peroxidase in the presence of H_2O_2 and leuco malachite green was given earlier (1): the reaction of the enzyme-substrate complex with leuco malachite green was shown to be in accord with this theory, not only from the standpoint of the rate of production of oxidized products (malachite green), but also from the standpoint of the reaction kinetics of the enzyme-substrate complex. Similar studies were made of the reaction of the enzyme-substrate complex with ascorbic acid but, at that time, no technique was available for a measurement of the overall reaction in terms of the rate of disappearance of substrate (H_2O_2) or acceptor (ascorbic acid).

Experimental

In this paper, two new methods have been utilized to measure the overall activity of peroxidase under the same conditions as the enzyme-substrate kinetics are measured. In the first case, a polarographic method is used to determine the rate of disappearance of H_2O_2 during enzymatic activity (a platinum microelectrode is polarized as an anode and inserted into the capillary observation tube of the rapid-flow apparatus). In the second case, the disappearance of ascorbic acid is measured in a rapid flow apparatus suitable for the recording of small changes of optical density in the ultraviolet region. By these two methods further proof is furnished over a wide range of conditions that the reaction kinetics of this enzyme are adequately described on the basis of the simple theory of enzyme-substrate complexes of Michaelis and Menten. Thus, the method for measuring the activity of peroxidase on the basis of measurements of the reaction kinetics of the enzyme-substrate complex is valid over a wide range of conditions.

Over a certain range of ascorbic acid concentration, the velocity

constant for the reaction of ascorbic acid with Complex II decreases with increasing ascorbic acid concentration and has afforded some basis for the belief that Complex II forms a ternary complex with ascorbic acid (2). These data permit a new interpretation of this effect.

The direct oxidation of ascorbic acid by peroxidase and H_2O_2 has been doubted; statements have been made that ascorbic acid is only oxidized *via* the cyclic oxidation and reduction of polyphenols and their quinone forms (4,5). These data clearly show that ascorbic acid can be oxidized in both ways: by direct reaction with Complex II or by reaction with the oxidation products of phenols, *etc.*, which have reacted with Complex II.

Peroxidase has been shown to form primary and secondary complexes with methyl or ethyl hydrogen peroxide as well as with H_2O_2 (3). Measurements of the reaction of these 3 complexes with ascorbic acid give nearly identical velocity constants; the activity of the peroxidase-peroxide complex is not decreased by the use of the substituted hydrogen peroxides.

A Direct Correlation between the Kinetics of the Enzyme-Substrate Complex of Peroxidase and the Rate of Disappearance of H_2O_2

In previous studies, no method was available for measuring the rate of disappearance of H_2O_2 at the same time as the kinetics of peroxidase were measured spectrophotometrically (1); the rate of production of malachite green from leuco malachite green was recorded. Recently it has been found that the platinum microelectrode polarized anodically can be used for the measurement of the disappearance of H_2O_2 (6,7). Such an electrode has been inserted into the capillary of the rapid-flow apparatus in such a way that it does not interfere with the spectrophotometric measurements. By means of this technique, it has been shown that the kinetics of H_2O_2 disappearance, as measured by the platinum microelectrode, agree very exactly with the rate of production of malachite green measured spectrophotometrically, the reaction being catalyzed by peroxidase (7). This is a satisfactory test of the electrode's performance.

Ascorbic acid is more suitable for studies of the kinetics of the enzyme-substrate compound of peroxidase over a wide range of experimental conditions because no colored oxidation products are formed which would obscure the spectrophotometric measurements and has, therefore, been used extensively. We can now show that the activity of peroxidase, calculated directly from the rate of disappearance of H_2O_2, as measured by the platinum microelectrode, agrees closely with the values of activity measured from the kinetics of the enxyme-substrate compound of peroxidase.

A typical record of enzyme-substrate (middle trace) and H_2O_2 (bottom trace) kinetics is shown in Fig. 1. At the start of the record, the flow is stopped and the observation tube of the rapid flow apparatus is filled with free peroxidase after the completion of a previous identical experiment in which the H_2O_2 was used up due to the

excess of ascorbic acid. Upon starting the flow, as shown by the downward deflection of the top trace, fresh peroxidase is mixed with fresh H_2O_2 and an excess of ascorbic acid. The abrupt downward deflection of the spectrophotometric trace shows the rapid formation of the enzyme-substrate compound. The slower fall of the platinum microelectrode trace shows the filling of the observation tube with fresh H_2O_2. The response of the electrode circuit is not as fast as that of the spectrophotometric trace

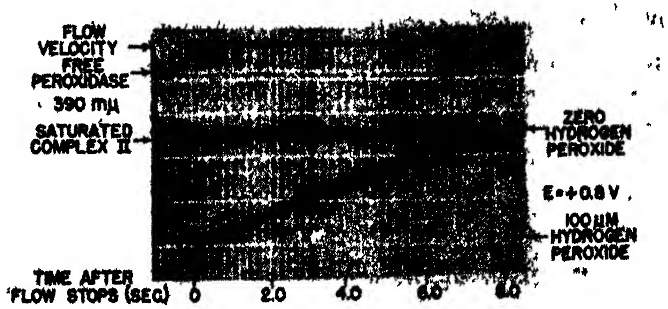

FIG. 1. Illustrating the correlation between the zero-order disappearance of H_2O_2 as measured by the platinum microelectrode and the constant steady-state concentration of the secondary peroxidase-hydrogen peroxide complex as measured spectrophotometrically at 390 mμ. 0.65 μM horseradish peroxidase, 100 μM H_2O_2, 2.4 mM ascorbic acid, pH = 4.7, 0.01 M acetate (Expt. 103).

but is adequate for these purposes. As the flow stops, longer times are measured and the disappearance of H_2O_2 caused by the activity of peroxidase is indicated by the linear rise of the bottom trace. The concentration of the enzyme-substrate compound remains in a steady state at its saturation value until the H_2O_2 concentration falls to a very low value. Then the steady state is terminated and the concentration of the enzyme-substrate compound falls to zero. The experimental conditions were intentionally chosen to demonstrate the classical saturated Michaelis compound which gives the zero-order kinetics of substrate disappearance.

To compute the activity of peroxidase from these kinetic data, the rate-determining reactions are written:

$$E + S \xrightarrow{k_1} ES \tag{1}$$

$$ES + AH_2 \xrightarrow{k_4} E + A + SH_2 \tag{2}$$

ES represents Complex II; although Complex II is preceded by Complex I, the latter is transformed into Complex II as rapidly as it is formed. The velocity constant, k_4, is taken as the measure of enzymatic activity toward the particular acceptor (AH_2) employed.

It has previously been shown that k_4 may be calculated from the enzyme-substrate kinetics (1,3) as follows:

$$k_4 = \frac{k_3}{[AH_2]} = \frac{x_0}{p_{max}\, t_{\frac{1}{2}\,off}\, [AH_2]}, \tag{3}$$

x_0 = initial H_2O_2 concentration.
p_{max} = maximum concentration of the enzyme-substrate complex during the cycle (= the enzyme concentration in Fig. 1 because the enzyme is saturated with substrate).
$t_{\frac{1}{2}\,off}$ = the time for the concentration of the enzyme-substrate complex to fall from maximum to half-maximum concentration.

k_4 is also calculated from the kinetics of disappearance of substrate (2):

$$k_4 = \frac{k_3}{[AH_2]} = \frac{dx}{dt} \cdot \frac{1}{p_{max}\,[AH_2]}, \tag{4}$$

where $\frac{dx}{dt}$ is the rate of disappearance of H_2O_2 as determined by the platinum microelectrode (Fig. 1) or the rate of disappearance of ascorbic acid as measured spectrophotometrically (Fig. 2).

It is possible to measure $\frac{dx}{dt}$ from the half-time of the kinetics of disappearance of substrate provided $\frac{dx}{dt}$ is constant (p is substantially constant) over this interval. Then dx/dt is simply $x_0/2\,t_{\frac{1}{2}}$. In that case, Eq. 4 simplifies as follows:

$$k_4 = \frac{k_3}{[AH_2]} = \frac{x_0}{2\,t_{\frac{1}{2}}} \cdot \frac{1}{p_{max}\,[AH_2]} \tag{5}$$

If the value of $t_{\frac{1}{2}\,off}$ in Eq. 3 is twice that of Eq. 5, then the two values of k_4 are the same. This provides a simple test for a Michaelis intermediate: The half-time of the enzyme-substrate cycle should be twice the half-time of the overall reaction provided the saturation of the enzyme-substrate complex is constant for the latter interval. And in all cases the overall reaction comes to a halt when the concentration of Complex II falls to zero.

The values of k_4 computed from Fig. 1 are 0.86 and $0.91 \times 10^4\ M^{-1} \times sec^{-1}$ calculated by Eqs. 3 and 4, respectively. Similar experiments have been carried out over a range of enzyme concentrations from 0.07 to 3.5 μM and ascorbic acid concentrations from 0.12 to 2.5 mM. The data cover a range from 0.6 to 78 μM H_2O_2/sec. Again the values of k_4, measured by the two methods, are found to be in satisfactory agreement.

In these particular reactions the enzyme was saturated with substrate; $p_{max} = e$ in Eqs. 3 and 4. Thus, the two determinations of k_4 are independent. In other cases, where $p_{max} < e$, the determinations are, to some extent, interdependent.

A Correlation between the Kinetics of Complex II and the Rate of Disappearance of Ascorbic Acid

Recent improvements in electronic techniques and in the design of the rapid-flow apparatus now permit the recording of kinetics of rapid reactions in the ultraviolet region. With suitable modifications of the stabilization circuits for the hydrogen discharge tube of the Beckman spectrophotometer, the fluctuations have been reduced to considerably less than one part in 1000. And with further modifications, the stability

and speed of response of the photoelectric circuit can be improved so that an optical density change of considerably less than 0.001 can be measured with a response time of less than 0.2 sec. A special mixing chamber and observation tube of a 10 mm. optical path and 1 mm. width has been fitted with silica end plates to permit measurement in the ultraviolet region. Since the data presented here are restricted to slower reactions which may be measured by the "stopped-flow" method, detailed data on the method of mixing and on the efficiency of mixing are not given in this paper. For this reason, the flow velocity trace is omitted in the records given in this paper.

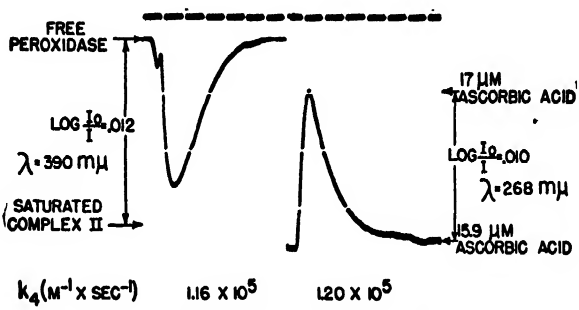

FIG. 2. Illustrating the correlation between the disappearance of ascorbic acid as measured spectrophotometrically at 268 mμ and the kinetics of Complex II measured at 390 mμ. The values of k_4 are computed according to Eqs. 3 and 4. 0.37 μM horseradish peroxidase, 1.1 μM H_2O_2, 17 μM ascorbic acid, pH = 4.6, 0.01 M acetate (Expt. 409b). The interval between markers is one second. The cell depth is 1 cm.

Fig. 2 shows recordings of the kinetics of the enzyme-substrate complex measured at 390 mμ and of the disappearance of ascorbic acid measured at 268 mμ. At 390 mμ, the explanation given above for the corresponding trace of Fig. 1 applies here. However, the two curves differ significantly; in Fig. 2, the concentration of Complex II does not reach its saturation value marked on the figure because the ratio of ascorbic acid to H_2O_2 concentration is fairly large. Since the records at 390 and 268 mμ can only be made by altering the wavelength setting of the monochromator, simultaneous recordings cannot be presented as in Fig. 1 and the same reaction is repeated at the two wavelengths. At 268 mμ, the abrupt *rise* of the trace indicates the injection of fresh ascorbid acid and H_2O_2 into the observation chamber. In accordance with the convention used in these recordings, increases of optical density are registered as upward deflections. The disappearance of ascorbic acid is indicated by the linear fall of the trace. It should be noted that this trace is inflected more than the corresponding trace of Fig. 1 because the concentration of Complex II begins to fall after the first second of reaction. The values of k_4 are calculated by Eqs. 3 and 4 and are in good agreement. In this case, however, the values of p_{max} used in Eq. 4 is that measured from the curve at 390 mμ.

The amount of ascorbic acid oxidized by the enzyme-substrate complex is readily computed from the amplitude of the trace at 268 mμ on the basis that the extinction coefficient of ascorbic acid is 10 cm.$^{-1}$ $\times$ mM^{-1} at pH 4.6 (9.35 cm^{-1} $\times$ mM^{-1} (8) is given elsewhere but the value of pH is not specified) and one ascorbic acid molecule is oxidized by one H_2O_2 molecule.

Both the polarographic and spectrophotometric methods for measuring the overall reaction of peroxidase with H_2O_2 and ascorbic acid give reaction velocity constants which are in good agreement with those obtained from direct spectroscopic measurements of the kinetics of the secondary peroxidase-H_2O_2 complex. This agreement indicates that this secondary complex is the Michaelis intermediate compound or the rate-determining intermediate in these reactions. Thus, the previous data obtained by a comparison of the kinetics of malachite green production with the kinetics of Complex II are verified and extended (1).

The Relation between Ascorbic Acid Concentration and the Value of k_4

It is to be noted that the values of k_4 (1.2×10^6 $M^{-1} \times$ sec^{-1}) obtained in Fig. 2 at lower ascorbic acid concentrations (17 μM) are much larger than those measured at

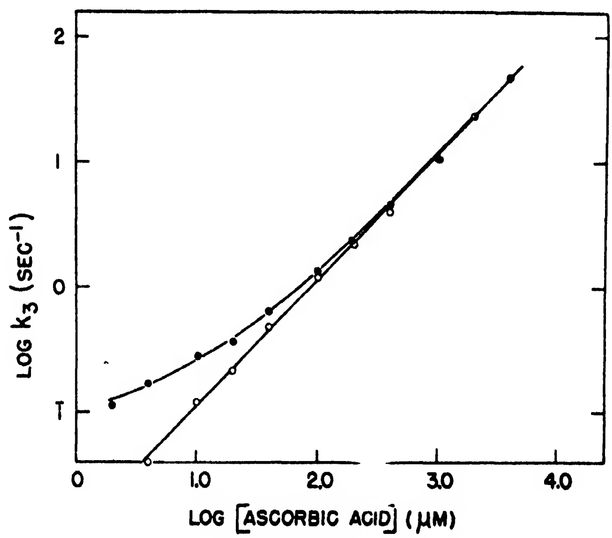

FIG. 3. The relation between the turnover number of peroxidase, k_3, of Eq. 3 and ascorbic acid concentration based upon measurements of the kinetics of Complex II. The results are plotted on a logarithmic scale. Experimental points, solid circles, experimental points corrected for a "blank" rate of k_3 = 0.16 sec^{-1}, open circles. 1.4 μM horseradish peroxidase, the ratio of ascorbic acid to H_2O_2 concentration is roughly 2, λ = 400 mμ, pH = 4.7, 0.01 M acetate buffer (Expt. 368).

larger ascorbic acid concentrations (2400 μM) in Fig. 1 ($0.9 \times 10^4\ M^{-1} \times sec^{-1}$). The values obtained in Fig. 2 are, in fact, in good agreement with the values ($1.8 \times 10^5\ M^{-1} \times sec^{-1}$) given in an earlier paper at approximately the same ascorbic acid concentration (1).

Since such a decrease of k_4 with ascorbic acid concentration might suggest that the turnover number is limited by the formation of a ternary complex of peroxidase, H_2O_2, and ascorbic acid, the relatiòn between the value of k_4 and ascorbic acid concentration has been investigated over a wide range. The values of k_3 have been computed according to Eq. 3 from measurementsof the values of p_{max} and $t_{\frac{1}{2}\,off}$ from the kinetics of Complex II. These values of k_3 are plotted against the ascorbic acid concentration on a logarithmic scale in Fig. 3. The experimental data (solid circles) fall along a curve which converges with a 45° straight line above 100 μM ascorbic acid. This shows that the high values of k_4 obtained at low ascorbic acid concentration decrease and become constant above 100 μM ascorbic acid.

It is assumed that the "spontaneous" breakdown of Complex II is $k_3 = 0.16\ sec^{-1}$, *i.e.*, that the "blank" rate of disappearance of H_2O_2 is $k_3 = 0.16\ sec^{-1}$ and this value is subtracted from the measured values of k_3 to give the corrected values of k_3 plotted as open circles in Fig. 3. It is now seen that the corrected data fall on a straight line at 45° over the whole range.

Thus, the reaction of Complex II with ascorbic acid is clearly of the second order over the measurable range. Certainly the constancy of k_4 over this wide range gives no evidence for the existence of a measurable

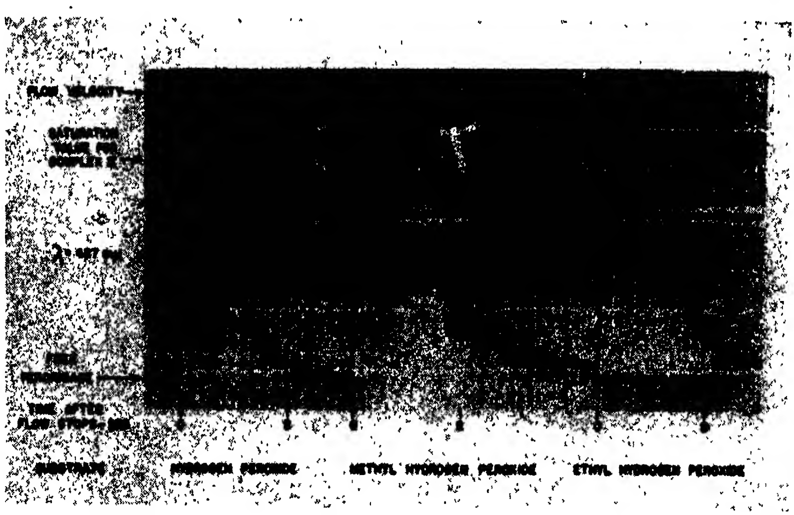

FIG. 4. Illustrating the reaction kinetics of the secondary complexes of peroxidase with H_2O_2, methyl hydrogen peroxide, and ethyl hydrogen peroxide in the presence of ascorbic acid. The peroxide concentrations are 3.6, 4.2, and 3.2 μM, respectively. The ascorbic acid concentration is 40 μM. 2.6 μM horseradish peroxidase, pH = 4.7, 0.01 M acetate = 427 mμ (Expt. 175a).

amount of a ternary peroxidase-H_2O_2-ascorbic acid complex. The "blank" rate, however appears to be exceptionally high—usually the blank rate is only 0.01 sec^{-1} and an explanation is provided on p. 400.

The Reaction of the Enzyme-Substrate Complexes of Peroxidase and the Alkyl Hydrogen Peroxides with Acceptors

In Fig. 4, the kinetics of the secondary complexes of peroxidase with H_2O_2, methyl hydrogen peroxide, and ethyl hydrogen peroxide are compared. The short lifetime of all 3 complexes indicates their rapid reaction with ascorbic acid. Since H_2O_2 combines most rapidly with peroxidase, the saturation of Complex II is highest, and, therefore, the "cycle" is shortest (see Eq. 3). And for this reason the saturation of the ethyl hydrogen peroxide complex is greater and its half-time smaller than that of the methyl hydrogen peroxide complex. The values of k_3 are readily calculated from Eq. 3, and the Michaelis constants [k_3/k_1 see Eq. 14 of Ref. (3)] are 1.3×10^{-7} M for H_2O_2, 9×10^{-7} M for methyl hydrogen peroxide, and 3.3×10^{-7} M for ethyl hydrogen peroxide.

TABLE I

The Activity of the Complexes of Horseradish Peroxidase with Hydrogen Peroxide, Methyl Hydrogen Peroxide, and Ethyl Hydrogen Peroxide towards Ascorbic Acid

2.6 μM horseradish peroxidase, pH = 4.7, 0.01 M acetate, λ = 427 mμ. (Expt. 175 a.)

Substrate................		Hydrogen peroxide		Methyl hydrogen peroxide		Ethyl hydrogen peroxide	
Concentration	μM	3.6	36	4.2	42	3.1	31
Ascorbic acid	μM	40	100	40	100	40	100
Oscillograph deflection ($p = e = 90$ mm.)	mm.	88	89	64	89	73	85
Half-time for decomposition of complex ($t_{\frac{1}{2}\,off}$)	sec	1.06	11	1.75	13	1.34	10.5
$k_3 = \dfrac{x_0}{p_{max}\, t_{\frac{1}{2}\,off}}$	sec^{-1}	1.34	1.26	1.30	1.26	1.1	1.2
$k_4 = \dfrac{k_3}{[\text{Ascorbic acid}]}$	$M^{-1}\,sec^{-1} \times 10^{-4}$	3.3	1.3	3.3	1.3	2.8	1.2

The values of k_4 are summarized in Table I. It is seen that the reaction velocity of the peroxidase-peroxide complex with ascorbic acid is very nearly independent of whether a hydrogen atom, a methyl group, or an ethyl group is attached to the peroxide group. This result is in rather striking contrast to the conclusion of Wieland and Sutter (9) that the activity of peroxidase with ethyl hydrogen peroxide is too small to be of any importance. In their test, the difference of activity between H_2O_2 and ethyl hydrogen peroxide was caused by the lower saturation of the peroxidase-ethyl hydrogen peroxide complex.

TABLE II

The Activity of the Complexes of Horseradish Peroxidase with Hydrogen Peroxide, Methyl Hydrogen Peroxide, and Ethyl Hydrogen Peroxide towards Ascorbic Acid

0.11 μM horseradish peroxidase, 2000 μM ascorbic acid, pH = 7.0, $\lambda = 427$ mμ. (Cuvettes—Expt. 280 b.)

Substrate	Hydrogen peroxide		Methyl hydrogen peroxide			Ethyl hydrogen peroxide	
Concentration—μM	4	8	2.6	5.2	10	2	8
Recorder deflection ($p = e = 22$)	16	22	7	7	11	7	17
Half-time for decomposition of complex ($t_{\frac{1}{2}\,off}$) sec	10	10.5	17	23	30	13	23
$k_3 = \dfrac{x_0}{p_{max}\, t_{\frac{1}{2}\,off}}$ sec^{-1}	5.0	6.9	4.3	6.8	6.1	4.4	4.1
$k_4 = \dfrac{k_3}{[\text{Ascorbic acid}]}$ M^{-1} sec$^{-1} \times 10^{-4}$	2.5	3.0	2.2	3.4	3.1	2.2	2.1
Average k_4 M^{-1} sec^{-1}	2800		2800			2200	

The comparison of the activities was repeated as shown in Table II at pH = 7.0 and with dilute enzyme and stronger ascorbic acid to ensure that the agreement in Table I was not due to a fortuitous choice of experimental conditions. The equality of the activity of the complexes is confirmed, although the values of k_4 are smaller at this pH.

Since this equality could be a particular property of the acceptor employed instead of an intrinsic property of the enzyme-substrate complex, both pyrogallol and guaiacol have been used as acceptors and again the 3 enzyme substrate complexes are found to have very nearly the same activity as shown in Table III.

TABLE III

The Activity of the Complexes of Horseradish Peroxidase with Hydrogen Peroxide, Methyl Hydrogen Peroxide, and Ethyl Hydrogen Peroxide with Pyrogallol and Guaiacol

2.6 μM horseradish peroxidase, λ = 427 mμ. (Expts. 180, 175 b.)

pH	6.5	6.5	6.5	4.6	4.6	4.6
Substrate	Hydrogen peroxide	Methyl hydrogen peroxide	Ethyl hydrogen peroxide	Hydrogen peroxide	Methyl hydrogen peroxide	Ethyl hydrogen peroxide
Concentration μM	3.6	4.1	3.1	3.6	4.1	3.1
Acceptor	Pyrogallol	Pyrogallol	Pyrogallol	Guaiacol	Guaiacol	Guaiacol
Concentration μM	20	20	20	20	20	20
Oscillograph deflection saturation value $p = e$ (mm.)	80	80	80	92	92	92
Oscillograph deflection (mm.)	60	28	37	82	42	48
Half-time for decomposition of complex ($t_{\frac{1}{2}\ \mathrm{off}}$) sec	0.45	1.1	0.71	0.60	1.3	0.66
$k_3 = \dfrac{x_0}{p_{max}\, t_{\frac{1}{2}\ \mathrm{off}}}$ sec^{-1}	4.1	4.1	3.6	2.6	2.6	3.5
$k_4 = \dfrac{k_3}{[\text{Acceptor}]}$ M^{-1} sec^{-1} $\times 10^{-5}$	2.1	2.1	1.8	1.3	1.3	1.7

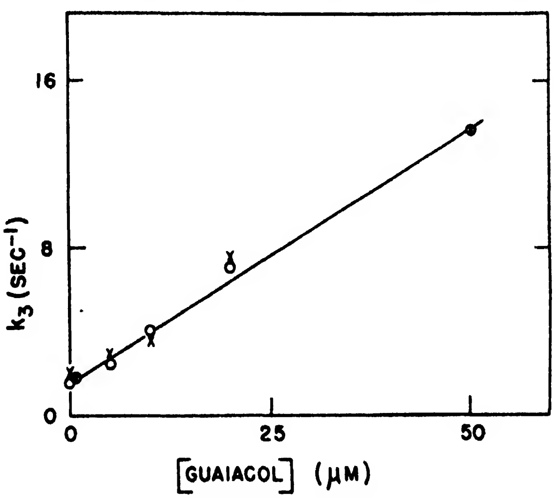

FIG. 5. The effect of guaiacol upon the turnover number of peroxidase measured from the kinetics of Complex II and computed according to Eq. 3 (circles), and upon the rate of disappearance of ascorbic acid measured at 290 mμ and computed according to Eq. 4. 0.4 μM horseradish peroxidase, 34 μM ascorbic acid, 10 μM H_2O_2, pH = 4.7, 0.01 M acetate (Expt. 408a).

The Effect of Phenols upon the Velocity Constant for the Reaction of Complex II and Ascorbic Acid

Although Fig. 3 clearly shows that a direct reaction between Complex II and ascorbic acid can occur, there is no reason to doubt that ascorbic acid can also be oxidized by reaction with quinones formed by the reaction of phenols with Complex II. For this reason, a study has been made of the effect of increasing amounts of guaiacol upon the value of the turnover number, k_3, as measured by the two methods of Fig. 2 and computed by Eqs. 3 and 4.

The data of Fig. 5 are in two parts. First, the circles are the values of k_3 computed from the kinetics of Complex II and represent the rate of disappearance of H_2O_2. This value would be expected to increase linearly with guaiacol concentration because Complex II is known to react with guaiacol. Second, the crosses represent the rate of disappearance of ascorbic acid measured in the ultraviolet region (290 mμ in this case) and the values of k_3 are computed according to Eq. 4. Since the enzyme is saturated with substrate ($p_{max} = e$), the computation of the values of k_3 by the two methods is independent. Therefore, no increase of the turnover number (k_3) for ascorbic acid would be expected unless there were some interaction between the oxidation of guaiacol and ascorbic acid, for example, a direct reaction between the products of guaiacol oxidation and the ascorbic acid.

There are at least two ways of showing that this direct reaction occurs: First, no tetraguaiacol formation has been observed as long as the ascorbic acid is in excess. This indicates that the quinone form of guaiacol reacts rapidly with ascorbic acid and is not free to form tetraguaiacol. Second, an amount of guaiacol or other phenol less than the initial amount of H_2O_2 sustains the accelerated turnover of ascorbic acid. This indicates that the quinone is reduced to the phenol on reaction with ascorbic acid and may again be oxidized by Complex II in a cyclic manner.

The first effect is clearly demonstrated in Fig. 6 which represents the reaction of Complex II with guaiacol in the presence (a) and absence (b) of ascorbic acid. In (a), at 298 mμ, the filling of the observation chamber with fresh ascorbic acid causes the rapid upward deflection of the trace which then linearly returns to the base line due to the oxidation of ascorbic acid. The record at 410.5 mμ in this case represents a control; no measurable reaction is seen because this wavelength is an isosbestic point between peroxidase and Complex II. However, in (b), where ascorbic acid is omitted, the formation of a tetraguaiacol is measured at this wavelength. The observation tube, in this case, is initially filled with the products of a previous identical experiment and injection of fresh reactants causes these products to be replaced by peroxidase, H_2O_2, and guaiacol. The rise of the trace indicates the formation of tetraguaiacol at roughly the same speed as ascorbic acid was shown to disappear in record (a) at 298 mμ. Record (b), at 298 mμ, shows that only the formation of tetraguaiacol is measured in the absence of ascorbic acid.

Thus, Fig. 6 shows that tetraguaiacol is not produced by the reaction of Complex II with guaiacol in the presence of ascorbic acid and suggests that the quinone form of guaiacol reacts directly with ascorbic acid.

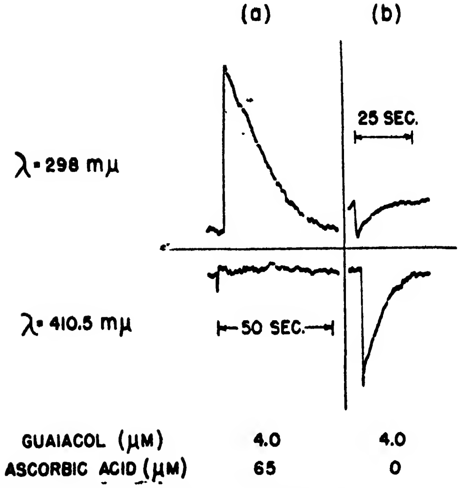

FIG. 6. Illustrating the suppression of tetraguaiacol formation in the reaction of Complex II with guaiacol in the presence of ascorbic acid. 0.4 μM horseradish peroxidase, 40 μM H_2O_2, pH = 4.7, 0.01 M acetate (Expt. 410b).

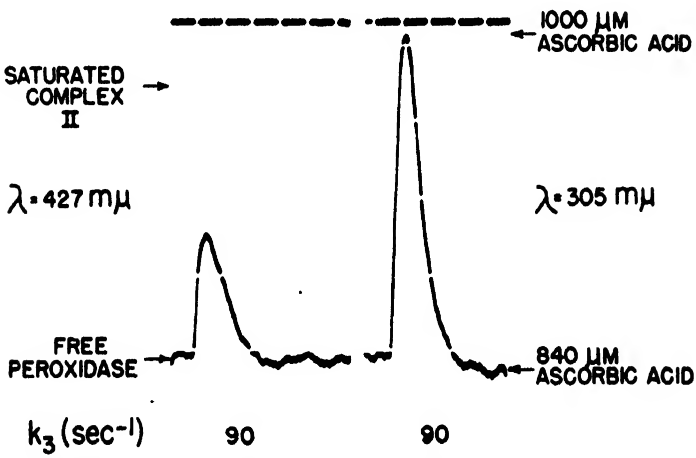

FIG. 7. Illustrating the accelerated turnover of a large amount of ascorbic acid in the presence of a small amount of a phenol. 0.4 μM horseradish peroxidase, 200 μM H_2O_2, 1000 μM ascorbic acid, 10 μM *p*-hydroxydiphenyl, pH = 4.7, 0.01 M acetate buffer (Expt. 410c). Time markers are 1 sec. interval.

It is possible to demonstrate the oxidation of a large amount of ascorbic acid by a small amount of phenol by using the conditions represented in Fig. 7. For a clean-cut demonstration, the H_2O_2 concentration is raised to 200 μM and the ascorbic acid concentration to 1000 μM. A highly reactive phenol, *p*-hydroxydiphenyl is used, so that the turnover caused by only 10 μM *p*-hydroxydiphenyl will be much greater

than that caused by the 1000 μM ascorbic acid. The records of Fig. 7 show, at 427 mμ, that the 200 μM H_2O_2 has reacted at a turnover number (k_3 computed according to Eq. 3) of 90 sec^{-1}, compared with 17 sec^{-1} measured in a separate experiment in the absence of *p*-hydroxydiphenyl. The record of Fig. 7 at 305 mμ verifies that the ascorbic acid has disappeared at about the same rate as the peroxide, k_3 = 90 sec^{-1}, based on the initial value of 200 μM H_2O_2.

Thus, the phenol reacts in a cyclic manner in the presence of ascorbic acid; the phenol is oxidized by Complex II to the quinone form, which oxidizes ascorbic acid and is thereby reduced to phenol which, in turn, is oxidized by Complex II. The reaction mechanism under these circumstances bears a certain resemblance to the system used by Nelson and Dawson for measuring tyrosinase activity (10).

It is of some interest to note in Fig. 7 that, at high ascorbic acid concentrations, the spectroscopically measured decrease of ascorbic acid concentration (160 μM using ϵ_{305} = 0.12 cm^{-1} $\times$ mM^{-1} for ascorbic acid) is somewhat less than the initial H_2O_2 concentration. This discrepancy is not yet explained but does not, however, affect the conclusion that cyclic oxidation and reduction of the phenol occurs in this reaction.

In this connection, it is desirable to know what type of oxidation product of phenols is required to react rapidly with ascorbic acid. With guaiacol, it is probable that it is the quinone form that reacts with ascorbic acid. But peroxidase-peroxides oxidize phenols which do not form quinones, for example, hydroquinone monomethyl ether, and, in this case, the primary oxidation product would probably be a free radical. Fig. 8 illustrates that the disappearance of ascorbic acid is accelerated by the product of the oxidation of hydroquinone monomethyl ether. The velocity constant for the reaction of Complex II with hydroquinone monomethyl ether measured from the rate

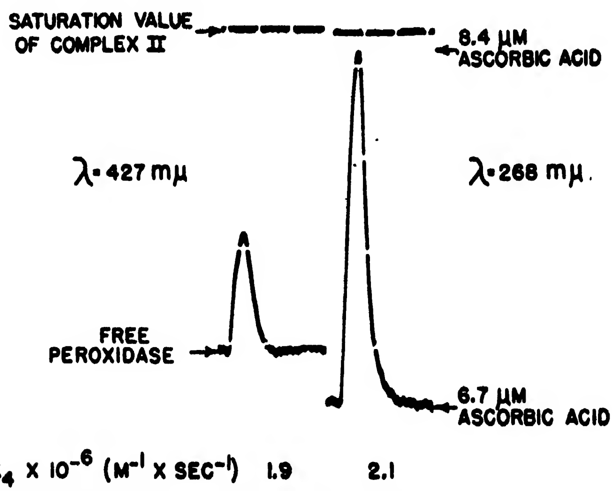

FIG. 8. Illustrating the accelerated turnover of ascorbic acid in the presence of hydroquinone monomethyl ether. The values of k_4 are computed for the reaction of Complex II with hydroquinone monomethyl ether. 0.4 μM horseradish peroxidase, 8.4 μM ascorbic acid, and 10 μM hydroquinone monomethyl ether, pH = 4.7, 0.01 M ascorbic acid. Time markers are 1 sec. interval (Expt. 410a).

of disappearance of ascorbic acid is 2.1×10^4 $M^{-1} \times \sec^{-1}$ and is in good agreement with the value measured in the absence of ascorbic acid from the kinetics of Complex II.

Thus, the cyclic oxidation can be promoted by phenols which are not known to form quinones and which presumably form free radicals in their reaction with Complex II.

It has already been shown in Fig. 3 that there is a direct reaction of Complex II and ascorbic acid. However, in the presence of phenols, the cyclic reaction occurs. If ascorbic acid reacts only with the oxidation product of the phenol and not with Complex II, the turnover of H_2O_2 would be independent of the ascorbic acid concentration.

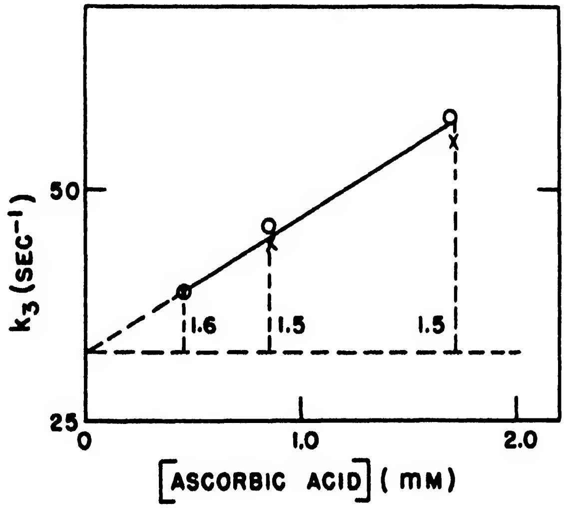

FIG. 9. Illustrating that the direct reaction of Complex II with ascorbic acid and the cyclic oxidation of ascorbic acid by the oxidation product of guaiacol occur simultaneously and without mutual interference. The circles represent the values of k_3 computed according to Eq. 3 from the kinetics of Complex II measured at 390 mμ. The crosses represent the values of k_3 computed according to Eq. 4 from the kinetics of ascorbic acid disappearance measured at 305 mμ. The increments of k_3 with respect to the extrapolated value are divided by the initial ascorbic acid concentration and are expressed as $k_4 \times 10^{-4}$. 0.4 μM horseradish peroxidase, 100 μM guaiacol, 200 μM H_2O_2, pH = 4.7, 0.01 M acetate (Expt. 409c).

Fig. 9 shows the results of an experiment designed to illustrate this effect in a quantitative manner. The reaction kinetics of Complex II are measured in the presence of a relatively large concentration of guaiacol (100 μM) so that the turnover number is relatively large ($\sim$ 30 sec^{-1}). The turnover number of this system (k_3) is then measured in the presence of 3 different concentrations of ascorbic acid and the data are computed according to Eq. 3. The results are plotted in Fig. 9 as circles. First, it is clearly shown that there is an increase of k_3 of only 1.5-fold for a 4-fold increase of ascorbic acid concentration. Similar results are obtained if the rate of disappearance of ascorbic

acid is measured at 305 mμ and the values of k_3 are calculated by Eq. 4 on the basis of 200 μM initial H_2O_2 concentration (crosses in Fig. 9). This small effect may be contrasted with the linear relation found in the absence of guaiacol as shown by Fig. 3.

Thus, the turnover number of peroxidase and H_2O_2 is relatively independent of ascorbic acid concentration in the presence of this relatively high concentration of guaiacol.

It is possible to explain in a quantitative manner the definite small increase in turnover with increasing ascorbic acid concentration that is shown in Fig. 9. Let us assume that ascorbic acid can react not only with the quinone produced by oxidation of guaiacol, but also by direct reaction with Complex II. Then the increase of k_3 caused by the reaction of Complex II with ascorbic acid can be computed for each of the values of ascorbic acid concentration by extrapolating the curve of Fig. 9 to zero ascorbic acid concentration (k_3 = 32 sec^{-1}) and measuring the three increments of k_3. If these increments are then divided by the appropriate ascorbic acid concentrations $\left(k_4 = \frac{k_3}{[\text{Ascorbic acid}]}\right)$ the values of k_4 (times 10^{-4}) are computed as shown on the figure and the average value is $1.5 \times 10^4\ M^{-1} \times$ sec.$^{-1}$. These values of k_4 may now be compared to those measured in Fig. 3 in this range of ascorbic acid concentration ($k_4 = 1.2 \times 10^4\ M^{-1} \times$ sec.$^{-1}$).

Thus, the direct oxidation of ascorbic acid by reaction with Complex II can occur whether phenols are present or not; when phenols are present, the direct reaction with Complex II as well as the reaction with the oxidation products of the phenols occurs.

The value of k_4 for the reaction of guaiacol with Complex II is given by dividing the extrapolated value of k_3 (32 sec.$^{-1}$) by the guaiacol concentration, and $k_4 = 3.2 \times 10^5$ $M^{-1} \times$ sec.$^{-1}$. If the data of Fig. 5 are now re-examined it is clearly seen that the value of k_4 may be obtained there from the increments of k_3 by the method described above. A value of $3 \times 10^5\ M^{-1} \times$ sec.$^{-1}$ is obtained, showing a good agreement between the two values. Thus, there is no measurable interaction between phenols and ascorbic acid in their reactions with Complex II.

Discussion

A total of 3 methods have thus far been used to correlate the overall activity of horseradish peroxidase and H_2O_2 with the kinetics of the enzyme-substrate complex. In the general reactions,

$$E + S \xrightarrow{k_1} ES \tag{6}$$

$$ES + AH_2 \rightarrow E + A + SH_2. \tag{7}$$

The rate of formation of product, A, was previously (1) measured by studies of the rate of formation of malachite green from leuco malachite green. In this paper, the rate of disappearance of the substrate, S,

was measured by a rapid polarographic method for the determination of H_2O_2 concentration. Also, the rate of disappearance of the reducing substrate or acceptor, AH_2, was measured by a rapid-flow apparatus suitable for the spectrophotometric measurements of ascorbic acid concentration in the region 268–305 mμ. In these 3 cases, the values of the reaction velocity constant of the enzyme-substrate complex with the reducing substrate or acceptor (k_4) are in close agreement with the values of k_4 computed from direct spectroscopic measuremnets of the kinetics of the enzyme-substrate complex according to the simple formula, $\frac{x_0}{p_{max}\, t_{\frac{1}{2}\,off}}$, previously derived from solutions of the equations representing reactions 6 and 7. This agreement confirms the fact that the secondary peroxidase-H_2O_2 complex is the rate-determining or Michaelis intermediate compound in peroxidase reactions.

The proofs of the intermediate complex theory that these experiments furnish allow us to reorient our definitions of the active catalyst in these reactions. The experimental data and the equations used to evaluate enzyme activity involve not measurements of the enzyme concentration but measurements of the concentration of the enzyme-substrate complex itself. This is the catalyst in these reactions, for their rate is proportional to its molar concentration.

These extensive experiments have been considered essential in order to justify the very wide use that has been made of the measurements, in terms of k_4, of the enzymatic activity of catalases and peroxidases based upon the kinetics of their enzyme-substrate complexes. This method of using the enzyme-substrate complex as the spectrophotometric indicator of the substrate concentration appears at present to be a valid method for the measurement of enzymatic activity over the range made possible by the present experimental methods.

In spite of statements to the contrary (4), the direct oxidation of ascorbic acid upon reaction with Complex II has been clearly demonstrated. The velocity constant for this reaction at pH = 4.6 is 1.9×10^4 $M^{-1} \times sec^{-1}$. With more dilute ascorbic acid, this velocity constant apparently increases up to 1.3×10^5 $M^{-1} \times sec^{-1}$, in accordance with the value given previously for dilute ascorbic acid, but these higher values are caused by an exceptionally high "blank" rate (see below). Thus, the smaller value is the correct velocity constant for the second-order reaction of Complex II and ascorbic acid.

This apparent variation of k_4 at the lower ascorbic acid concentrations is not explained by the formation of a ternary peroxidase-H_2O_2-ascorbic acid complex which would decrease k_4 only at higher ascorbic acid concentrations. In fact, these data show that the life-time of such a complex, if it exists, is less than 0.02 sec. under these experimental conditions (k_4 is constant at $1.2 \times 10^4\ M^{-1} \times \text{sec}^{-1}$ at 4 mM ascorbic acid: a turnover number of about 50 sec^{-1}).

Ascorbic acid is oxidized, not only by direct reaction with Complex II, but also by reaction with the oxidation products of phenols which have reacted with Complex II. This latter type of reaction was studied earlier and has been extensively used as a means for determining tyrosinase activity (10). Direct experiments show that a primary oxidation product of the phenol reacts with ascorbic acid; for example, the formation of tetraguaiacol in the oxidation of guaiacol is prevented. Albert and Falk (5) have shown recently that acridines promote a similar reaction with ascorbic acid, H_2O_2, and hematin. Also, the cyclic oxidation and reduction of the phenols is proved by experiments in which the oxidation of many equivalents of ascorbic acid per phenol molecule is demonstrated.

It is clearly shown that these two reaction mechanisms may occur simultaneously and without mutual interference; the total activity is the sum of the two separate activities. The absence of mutual interference in the reaction of Complex II with acceptors such as ascorbic acid or phenols supports the evidence above that any peroxidase-H_2O_2-acceptor complex must have too short a life to be of significance under these experimental conditions.

Experimental tests show that ascorbic acid is oxidized by the oxidation products of phenol that are known to form quinones or by phenols which are known not to form quinones. In the latter case, it is probable that the ascorbic acid is oxidized by reaction with a free radical formed on oxidation of the phenol.

No data are available on the speed of the reaction of ascorbic acid with such oxidation products of phenols. However, minimum values of the reaction velocity constant may be inferred (1) from the fact that tetraguaiacol formation is inhibited and (2) from the fact that the disappearance of ascorbic acid occurs at the same rate as the disappearance of H_2O_2. The first case gives us a value of $> 1.4 \times 10^5\ M^{-1} \times \text{sec}^{-1}$ because tetraguaiacol is formed as fast as guaiacol reacts with Complex II (11) and this reaction has a velocity constant of $1.4 \times 10^5\ M^{-1} \times \text{sec}^{-1}$.

The second case gives a value of $> 2 \times 10^6$ because, in Fig. 8, the rate of ascorbic acid disappearance follows the rate of H_2O_2 disappearance when the concentration of ascorbic acid is roughly equal to that of the hydroquinone monomethyl ether: the ascorbic acid must have reacted with the oxidation product of hydroquinone monomethyl ether more rapidly than the latter reacted with Complex II.

It is now possible to present an explanation for the exceptionally high values of the "blank" rate observed in the reaction of Complex II with ascorbic acid. It has been found (3) that peroxidase preparations, even though thoroughly dialyzed, appear to contain a small amount of an acceptor which accelerates the decomposition of Complex II. The amount of this acceptor appears to be of the same order of magnitude as the enzyme concentration and, therefore, is usually exhausted by the addition of a few equivalents of peroxide, after which the usual low value of the blank rate is obtained (see Ref. 3, Fig. 8). If, however, this acceptor acts in a cyclic manner, as do phenols, then, in the presence of an excess of ascorbic acid, the high "blank" rate would be sustained, even though a large amount of peroxide were added. This is apparently the case and the value of $k_3 = 0.16$ sec^{-1} found in Fig. 3 is caused by this acceptor substance causing the oxidation of ascorbic acid by the "cyclic" mechanism.

Since it is estimated that the amount of this acceptor is roughly equal to the enzyme concentration (1.4 μM in Fig. 3), the value of k_4 for this acceptor is very roughly $0.16/1.4 \times 10^{-6} = 1 \times 10^5 M^{-1} \times \text{sec}^{-1}$, a very reasonable value for peroxidase acceptors. Thus it appears that the acceptor substance associated with the horseradish peroxidase molecule is capable of both oxidation and reduction. Presumably this acceptor has a phenolic or an amino functional group. But its exact relation to the peroxidase protein is not yet known.

The velocity of the reaction of Complex II with ascorbic acid, pyrogallol and guaiacol is very slightly affected by whether H_2O_2, methyl hydrogen peroxide, or ethyl hydrogen peroxide is attached to peroxidase. It is probable that these values are equal to within the experimental error of these methods. Apparently the reactivity of the iron-peroxide bond is not appreciably altered by the alkyl substituent on the peroxide molecule. These experiments are in agreement with those on the reactions of the catalase-peroxide complexes with alcohols. In that case, nearly equal reaction velocity constants were measured (12).

Wieland and Sutter (9) found that the activity of peroxidase with ethyl hydrogen peroxide as a substrate is only 1/5 that found when H_2O_2 is used as a substrate. The smaller value of peroxidase activity can readily be explained because peroxidase has a lower affinity for ethyl hydrogen peroxide than for H_2O_2, and thus the concentration of Complex II, for equal peroxide concentrations, would be smaller. Nevertheless, the activity of Complex II toward ascorbic acid is the same. This is an example of the gross errors that arise in studies of enzyme activity in which the saturation of the enzyme with substrate is ignored.

CONCLUSIONS

1. Further evidence that the kinetics of the enzyme-substrate compound of peroxidase are in accord with the Michaelis theory has been obtained by a direct correlation of the spectroscopically measured kinetics of the enzyme-substrate complex (a) with the rate of disappearance of substrate (H_2O_2) as measured by the platinum microelectrode and (b) with the rate of disappearance of acceptor (ascorbic acid) as measured by ultraviolet spectrophotometry. The previous correlation with the rate of appearance of oxidized acceptor (malachite green) is, therefore, extended.

2. Ascorbic acid is oxidized by direct reaction with Complex II and the reaction of ascorbic acid and Complex II is of the second order over a very wide range of ascorbic acid concentrations. The reaction velocity constant is $1.2 \times 10^4\ M^{-1} \times \text{sec}^{-1}$ at pH = 4.7. No evidence for the existence of a ternary complex of peroxidase, H_2O_2 and ascorbic acid was obtained.

3. Ascorbic acid is also oxidized by an indirect reaction involving the cyclic oxidation of phenols by their reaction with Complex II and by their reduction by ascorbic acid. The velocity of this latter reaction is very high ($> 10^6\ M^{-1} \times \text{sec}^{-1}$) and the overall reaction velocity is determined by the speed of the reaction of Complex II with phenols.

4. The direct and indirect reaction are independent and occur simultaneously and without mutual interference.

5. Ascorbic acid is oxidized by the oxidation products of phenols that are known to form quinones or by phenols that are known not to form quinones. In the latter case, it is probable that ascorbic acid is oxidized by reaction with a free radical formed on the oxidation of the phenol.

6. The apparent rise of the velocity constant (k_4) for the reaction of Complex II with ascorbic acid at low concentrations of the latter is attributed to an indirect oxidation of ascorbic acid promoted by the "acceptor" present in peroxidase preparations and accounts for the higher value of k_4 obtained previously (1).

7. The velocity constant for the reaction of Complex II with ascorbic acid, pyrogallol, and guaiacol is affected only slightly by whether H_2O_2 or an alkyl hydrogen peroxide is attached to peroxidase. Wieland and Sutter found a much lower peroxidase activity with ethyl hydrogen peroxide as a substrate, but they ignored the partial saturation of enzyme with substrate.

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The Properties of the Enzyme-Substrate Compounds of Horseradish Peroxidase and Peroxides.[1] IV. The Effect of pH upon the Rate of Reaction Complex II with Several Acceptors and Its Relation to Their Oxidation-Reduction Potential

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Introduction

In previous papers a direct method of determining the speed of the reaction of the enzyme-substrate compound of peroxidase (Complex II) with reducing substrates or acceptors has been developed and tested in some detail (1). The velocity constant for the reaction of Complex II with the acceptor (k_4) is an absolute measure of the enzymatic activity toward a particular acceptor at a given temperature. In this paper, the effect of pH upon k_4 for several rather different acceptors is determined so that appropriate conditions for the survey of the values of k_4 for a large number of acceptors may be established for the experiments recited in the following paper. It has also been found that the values of k_4 may be satisfactorily measured over a wide range of pH values by these methods.

Theorell and Paul (2) have found that horseradish peroxidase contains 2 heme-linked groups of pK = 4.0 and 5.0, and they have shown that the effect of pH upon the rate of production of purpurogallin from pyrogallol in the presence of peroxidase and H_2O_2 accurately coincides with the heme-linked group of pK = 4.0. These studies have been carried out to show whether this decrease of activity can be verified by direct measurements of the value of k_4 based on spectrophotometric

[1] This is No. 11 of a series on catalases and peroxidases.

[2] John Simon Guggenheim Memorial Fellow (1946-1948).

measurements of the kinetics of the enzyme-substrate complex of peroxidase.

It is also of interest to determine whether results identical with those of Theorell and Paul would be obtained in the presence of other acceptors.

The velocity constant for the reaction of Complex II with pyrogallol has been evaluated and has been compared with the value computed for pure peroxidase on the basis of the P.Z. test.

The effect of pH upon the oxidation-reduction potential of several acceptors toward which peroxidase exhibits a high activity has been studied by Ball and Chen (3) and Ball (4). Thus, it is possible to measure the values of k_4 for the reaction of Complex II with these acceptors at various values of pH and thereby to determine the effect of oxidation-reduction potential upon k_4. This affords a convenient method for the investigation of a change of oxidation potential without changing the nature of the acceptor molecule.

Experimental

The Effect of pH upon k_4

The values for the reaction velocities of Complex II with the various acceptors are based upon measurements of the kinetics of Complex II and are computed by means of Eq. 3 of the preceding paper (1). The acceptors chosen for this study are selected as representative samples of the main types of peroxidase acceptors and include 3 types of phenols, the leuco base of a triphenylmethane dye, leuco malachite green, and an endiol structure, ascorbic acid. The 3 phenols are a monosubstituted monophenol, guaiacol (*o*-methoxyphenol), a diphenol, hydroquinone, and a triphenol, pyrogallol. The pH range of special interest is 6 to 3.8 because this includes the range in which Theorell and Paul have found heme-linked groups in horseradish peroxidase (pK = 4.0 and 5.0).

In 3 cases, colored oxidation products of the acceptors are formed in sufficient quantity to permit their measurement under the same conditions as the kinetics of Complex II are measured: leuco malachite green forms the green dye, malachite green, guaiacol forms the red tetraguaiacol, and pyrogallol forms the yellow purpurogallin. And, in the case of guaiacol and leuco malachite green, it is possible to compute values of k_4 from the rate of formation of their colored forms by means of Eq. 4 of reference (1). With purpurogallin a difficulty arises that is discussed later.

The results of all these experiments are given in Table I. Considering guaiacol first, it is seen that the values of k_4, as measured from the kinetics of Complex II at 390 mμ, are constant to within the experimental error from pH 4.3 to 6.7. At pH = 3.5 there is a slight decrease of activity. Similar constancy of k_4 is obtained in the pH region 4.3–5.3 based on measurements of the rate of production of tetraguaiacol at 540 mμ. There is some decrease of activity at pH = 3.5 and some increase at pH 6.7.

With hydroquinone, there is no great change of k_4 over the pH region studied. The velocity constant for the reaction of hydroquinone with Complex II is extremely high; it is within an order of magnitude of the velocity constant for the combination of peroxidase with H_2O_2. Therefore, the "cycle" of Complex II is extremely brief (~0.2 sec), and the saturation of enzyme with substrate is small and the accuracy of the data is not as high as for the other acceptors. The consistency of the data is good and is suitable for demonstrating the effect of pH upon k_4. The absolute values of k_4 are, however, preliminary.

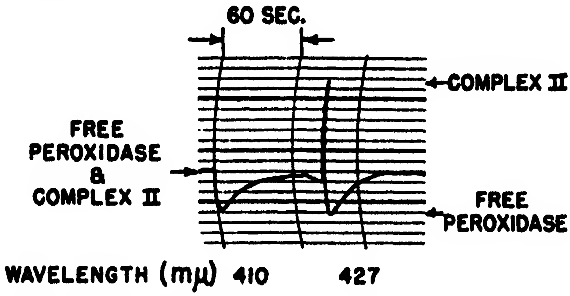

FIG. 1. Illustrating the discrepancy between the kinetics of Complex II and the production of colored oxidation products of pyrogallol. At 410 mμ the production of oxidation products only is shown, and at 427 mμ the formation and rapid disappearance of Complex II is shown to precede the formation of colored oxidation products of pyrogallol (Expt. 389).

With pyrogallol, nearly constant values of k_4 are measured from pH 3.5 to 5.3; an increase is shown at 6.7. The production of colored oxidation products in this reaction can readily be measured, but the nature of their reaction kinetics is strikingly different from that of Complex II. It was pointed out earlier (1) that a criterion for correspondence between the kinetics of Complex II and the overall reaction is that the half-time ($t_{\frac{1}{2}\,off}$) of the cycle of Complex II should be about twice that of the half-time of the overall reaction. And in all cases the overall reaction should come to a halt when the concentration of Complex II falls to zero.

Fig. 1 shows the relation between the reaction kinetics of Complex II and the formation of colored oxidation products of pyrogallol. At 410 mμ the latter reaction alone is measured, since the peroxidase and the Complex II spectra have an isosbestic point at this wavelength. The observation tube is initially filled with the products of a previous identical experiment which, upon initiation of the flow, as indicated by the abrupt fall of the trace, are replaced by unreacted preoxidase, H_2O_2, and pyrogallol. The slow upward rise of the trace indicates the increase of optical density caused by the formation of oxidation products of pyrogallol.

At 427 mμ the kinetics of the formation and disappearance of Complex II are recorded, as well as the production of colored oxidation products of pyrogallol. The observation tube is filled with the products of a previous experiment and initiation of the flow replaces these products with fresh reactants. The rapid formation of Complex II is indicated by the abrupt upward deflection of the trace. In a few seconds, the turnover of substrate is complete and the concentration of Complex II falls to zero. Instead of the formation of colored products being complete at this time, their formation has barely begun and this reaction proceeds for some time after the termination of the kinetics of Complex II as the slow rise of the trace clearly shows.

Thus, the production of colored substance (presumably purpurogallin) recorded here does not occur directly on reaction of pyrogallol with Complex II and is an unreliable indication of peroxidase activity under these conditions. That purpurogallin is not directly formed from pyrogallol is well known (5), and it is probable under these experimental conditions that one of the several steps involved in the production of colored oxidation products is slower than the rate at which pyrogallol reacts with Complex II. For this reason the values of k_4 for the reaction of pyrogallol and Complex II are based solely upon the measurements of the reaction kinetics of Complex II and, as these data show, are substantially independent of pH over the interval of particular interest.

With leuco malachite green, the values of k_4 are constant only in the relatively narrow pH range, 4.3–4.7. In more acid solutions k_4 decreases. However, fair agreement between the kinetics of Complex II measured at 390 mμ and the kinetics of production of malachite green at 550 mμ is obtained over the pH range 3.4–4.7, and the value of k_4 measured at pH = 4.7 is in good accord with the value measured previously (6). Above pH 5.3, two changes in the reaction occur. First, the values of k_4 measured from the kinetics of Complex II decrease significantly: about 10-fold from pH 4.7 and 6.7. Second, the amount of malachite green produced decreases markedly: over 6-fold between pH 4.7 and 6.7. Since the same amount of peroxide is utilized in each experiment, the data indicate that the leuco base is oxidized by Complex II not only to the dye, but, at higher values of pH, to a colorless form, presumably the colorless carbinol base of the triphenyl methane. Apparently this change in the course of the reaction alters the value of k_4.

With ascorbic acid, the value of k_4 is measured from the kinetics of Complex II at 390 mμ and is seen to be constant to within the experimental error between pH 3.5 and 5.3.

Above pH 5.3 there is a striking decrease of k_4. This effect is attributed to a change in the ascorbic acid molecule. The value of pK for the ascorbic acid-ascorbate ion system is 4.21 (4), and this change of ionization may cause the decrease of k_4. Other changes in the molecule may be involved, because k_4 does not decrease markedly until the pH is above 5.3. Some of the possible forms of ascorbic acid are discussed by Ball (4).

These experiments clearly show that the velocity of the reaction of Complex II and a selected group of acceptors is very little affected by pH in the region 3.5–5.3 which covers the range where Theorell and Paul have found two heme-linked groups in peroxidase (at pH = 4.0

TABLE

The Activity of Horseradish Peroxidase and

2.6 μM horseradish peroxidase. pH 3.5–5.3 in 20 mM acetate

	pH	3.4	4.3	4.7	5.3	6.7	3.4	4.3	4.7	5.3	6.7
	Acceptor	Ascorbic acid					leuco malachite green				
	Concentration μM	2000					10				
	H_2O_2 Concentration—μM	90					3.6				
Enzyme substrate kinetics $\lambda =$ 390 mμ	p_{max} (μM)	1.8	1.9	2.1	2.0	2.2	2.6	2.6	2.6	2.6	2.6
	Half-time for decomposition of complex —sec.	1.2	1.5	1.4	2.0	8.4	1.4	0.63	0.69	1.8	5.7
	$k_3 = \frac{x_0}{p_{max}\, t_{\frac{1}{2}\,off}}$ sec^{-1}	41.5	31	31	23	4.9	1.0	2.2	2.0	0.77	0.24
	$k_4 = \frac{k_3}{(\text{Acceptor})}$ M^{-1} sec^{-1} $\times 10^{-4}$	2.1	1.6	1.6	1.2	0.25	10	22	20	7.7	2.4
Overall reaction kinetics	λ mμ						550	550	550	550	550
	Oscillograph deflection due to oxidized acceptor—mm.						45	60	62	40	<10
	Rate of production of oxidized acceptor $\frac{dx}{dt}$ μM/sec						4.1	7.3	7.3	3.9	—
	$k_3 = \frac{dx}{dt}\frac{1}{p}$ sec^{-1}						1.6	2.8	2.8	1.5	—
	$k_4 = \frac{k_3}{(\text{Acceptor})}$ $M^{-1} \times$ sec^{-1} $\times 10^{-4}$						16	28	28	15	—

and 5.0). In the two cases where the values of k_4 definitely change with pH, evidence is available which indicates that

(1) The nature of the acceptor molecule changes with pH, and

(2) the nature of the oxidation products of the acceptor changes with pH.

In general, the lack of sensitivity of the reaction of Complex II with the acceptor molecule may be attributed to the stoichiometry of the reactions:

$$\text{Per OH} + H_2O_2 \overset{k_1}{\longleftrightarrow} \text{Per OOH} - \text{I} + H_2O \tag{1}$$

$$\text{Per OOH} - \text{I} \overset{k_7}{\longleftrightarrow} \text{Per OOH} - \text{II} \tag{2}$$

$$\text{Per OOH} - \text{II} + AH_2 \overset{k_4}{\longleftrightarrow} \text{Per OH} + \text{A} + H_2O, \tag{3}$$

I

H_2O_2 toward Various Acceptors at Various Values of pH

buffer, pH 6.7 in 5 m*M* phosphate buffer. (Expt. 171).

3.4	4.3	4.7	5.3	6.7	3.5	4.3	4.7	5.3	6.7	3.5	4.3	4.7	5.3	6.7
Hydroquinone					Guaiacol					Pyrogallol				
200					40					100				
90					9.0					18				
1.0	1.2	1.5	1.5	1.6	2.5	2.4	2.5	2.4	1.8	1.4	1.6	1.6	1.8	1.3
0.40	0.17	0.28	0.25	0.17	0.85	0.60	0.60	0.58	0.36	0.66	0.51	0.59	0.56	0.37
220	420	220	250	340	4.1	5.3	6.0	5.9	14	19	21	19	18	36
110	210	110	130	170	10	13	15	15	25	19	21	19	18	36
					540	540	540	540	540					
					30	30	33	30	30					
					7.5	17.5	17.5	25	25					
					2.9	6.7	6.7	9.6	13					
					7.3	17	17	24	33					

the net reaction being

$$H_2O_2 + AH_2 \rightarrow 2H_2O + A. \quad (4)$$

Within the pH range over which the activity of peroxidase is constant it is unlikely that hydrogen or hydroxyl ions are reaction products.

These experiments were not, however, carried far enough into acid solutions to determine where peroxidase activity falls rapidly. But the small, consistent decrease of k_4 for guaiacol, pyrogallol, and hydroquinone between pH 4.3 and 3.4 suggests that this decrease of activity may occur not far below pH = 3.4.

A Computation of the P.Z. of Horseradish Peroxidase from the Value of k_4

By these direct methods, the velocity of the reaction of Complex II and pyrogallol (k_4) is $3 \times 10^5\ M^{-1}\ sec^{-1}$ at pH = 6.7 and 25° C. (based on Table I and other data).

This may be converted into P.Z. as follows. From Eq. 4 of reference (1), the rate of disappearance of hydrogen peroxide $\left(\frac{dx}{dt}\right)$ is

$$\frac{dx}{dt} = k_4 a p_{max}, \tag{5}$$

where p_{max} is the steady-state concentration of Complex II and a is the pyrogallol concentration. Also, from reference (1) and (7),

$$p_{max} = \frac{xe}{x+K_{m2}} \tag{6}$$

and

$$K_{m2} = \frac{k_4 a}{k_1}. \tag{7}$$

By substituting for K_{m2} in Eq. 6 and for p_{max} in Eq. 5, and simplifying,

$$\frac{dx}{dt} = \frac{e}{\frac{1}{k_4 a} + \frac{1}{k_1 x}} \tag{8}$$

the values, $k_4 = 3 \times 10^6$, and $k_1 = 9 \times 10^6 M^{-1}\ \mathrm{sec}^{-1}$, are available. From the definition of P.Z., 1.25 g. pyrogallol, 12.5 mg. H_2O_2, and 1 mg. peroxidase are used in a volume of 500 cc. (8). Thus $a = 2 \times 10^{-2}\ M$, $x = 7.4 \times 10^{-4}\ M$, $e = 4.5 \times 10^{-8}\ M$. On substitution,

$$\frac{dx}{dt} = \frac{4.5\times10^{-8}}{\frac{1}{3\times10^6\times2\times10^{-2}} + \frac{1}{9\times10^6\times7.4\times10^{-4}}} = \frac{4.5\times10^{-8}}{3.2\times10^{-4}} = 1.4\times10^{-4}\ M\times\mathrm{sec}^{-1}.$$

P.Z. is not evaluated in moles of H_2O_2 per liter per sec, but in mg. of purpurogallin per 500 cc. per 5 minutes (8). Thus,

$$\mathrm{P.Z.} = 1.4\times10^{-4}\times\frac{220}{3}\times5\times60\times\frac{500}{1000}\times1000 = 1500, \tag{9}$$

remembering that 3 molecules of hydrogen peroxide are required to form 1 molecule of purpurogallin of molecular weight = 220.

Theorell, however, obtains a value P.Z. = 900 for his pure preparations (9) which is significantly less than the value of 1500 computed on the basis of k_4. A possible explanation is that production of purpurogallin lags the disappearance of H_2O_2 and pyrogallol, as has been already demonstrated under the experimental conditions of Fig. 1. In this case a smaller value of activity would be expected on the basis of measurements of the rate of purpurogallin production.

Recently, Ettori (10) has shown that the addition of phosphate buffer to the 500 cc. reaction mixture (see his Fig. 1) gives approximately a 1.5-fold increase of P.Z., a factor which would bring Theorell's value into fair agreement with our computed value of P.Z. = 1500.

One comment may be made upon Ettori's experiment in which the total volume of the reaction mixture was varied while the weights of the reactants were maintained constant.

By substituting for the concentrations of Eq. 8, as follows:

$$e = g_e/VM_e, \qquad a = g_a/VM_a, \qquad x = g_x/VM_x,$$

where g is the weight of the particular substance, M its molecular weight, and V the volume of the reaction mixture in liters, the following relation is obtained:

$$\frac{dx}{dt} = \frac{\frac{g_e}{M_e V}}{\frac{VM_a}{k_4 g_a} + \frac{VM_x}{k_1 g_x}} = \frac{1}{V^2}\left[\frac{\frac{g_e}{M_e}}{\frac{M_a}{k_4 g_a} + \frac{M_x}{k_1 g_x}}\right]. \tag{10}$$

Eq. 9 also involves the volume of the reaction mixture:

$$\text{P.Z.} = \frac{dx}{dt}\left[\frac{Mp}{3} \times 5 \times 60 \times V \times 1000\right] \tag{11}$$

Mp is the molecular weight of purpurogallin.

By substituting for $\frac{dx}{dt}$ its value in Eq. 10, the total effect of volume variation upon P.Z. is seen to be

$$\text{P.Z.} = \frac{1}{V}\left[\frac{\frac{g_e}{M_e}}{\frac{M_a}{k_4 g_a} + \frac{M_x}{k_1 g_x}}\right] Mp \times 10^5. \tag{12}$$

Thus P.Z. would be expected to vary in inverse proportion to the volume of the reaction mixture, a decrease of the volume from 500 to 50 cc. would be expected to increase the value of P.Z. 10-fold. But in Ettori's experiments (10) this change of volume caused an increase of P. Z. of only 1.8-fold (1000 to 1800 in phosphate buffer).

These computations clearly show that, although there is fair agreement between the computed and measured values fo P.Z. in the 500 cc. volume in the presence of phosphate, hugh discrepancies arise when the concentration of the reactants is increased by reducing the total volume of the reaction mixture. Although it is possible that k_4 actually decreases under these conditions, it is more likely that the slow step in the production of colored oxidation products of pyrogallol clearly shown in Fig. 1 is responsible for the discrepancies. In this case the high sensitivity of the P.Z. determination to minute traces of impurities, buffer concentration, and pH, and the anomalous behavior on reduction of the volume would not be unexpected. And such effects are

not characteristic of the primary reaction of Complex II with the acceptor molecule.

In general, the measurement of overall activity in terms of the formation of tetraguaiacol appears to be a much more reliable test for the activity of peroxidases, in accord with the conclusion of Mann (11). Wherever the techniques are available, the direct measurement of the kinetics of Complex II gives more incisive results and should be used.

The Relation between Oxidation-Reduction Potential of the Acceptor and the Velocity of its Reaction with Complex II

Since we are able, by these direct methods, to measure the velocity constant for the reaction of Complex II and the acceptor, it is desirable to examine what correlation exists between k_4 and the oxidation-reduction potential of the acceptor. This may be done in several ways: first, by a comparison of acceptors of different oxidation-reduction potentials with the values of k_4 at a particular pH; second, by comparing the variation of the oxidation-reduction potential of a particular acceptor with its values of k_4 at the various values of pH; and third, by varying the ratio of the oxidant to the reductant forms of the acceptor.

TABLE II

The Relation between the Velocity Constant for the Reaction of Complex II with Various Acceptors and Their Oxidation-Reduction Potential at pH = 4.7

Acceptor	$k_4(M^{-1} \times sec^{-1} \times 10^{-4})$	E (Refs. 3 and 4)
Catechol	230	0.510
Pyrogallol	21	0.431
Hydroquinone	250	0.411
Ascorbic acid	1.2	0.136

Table II gives some examples of the first case. The oxidation-reduction potentials (E) are given by Ball and Chen (3) and Ball (4) and correspond to pH = 4.7. First, the data show that acceptors having nearly the same oxidation-reduction potential have quite different values of k_4. This is especially striking in the comparison of hydroquinone and catechol with pyrogallol where an approximately 10-fold difference of k_4 exists. With ascorbic acid the values of E and k_4 are both smaller than with the phenols.

Since it is possible that the changes in structure of the acceptor are obscuring the relation between oxidation-reduction potential and k_4 as represented by the data of Table II, the comparison may be made for a particular acceptor at various values of pH. Ball and Chen have shown that the oxidation-reduction potential of the acceptors listed in Table II decreases at the uniform rate of 60 mV per unit increase of pH.

Thus, we can now refer to Table I, where the pH range from 3.5 to 6.7 corresponds to a change of oxidation-reduction potential of $-$ 192 mV, which corresponds to a change of roughly 50% of the value at pH = 3.5. In no case do we find a regular change of k_4 with pH and in those cases where a large change is found, a significant change in the nature of the oxidation product of the reaction or in the nature of the acceptor has probably occurred.

A similar constancy of k_4 with pH has been found in the reaction of catalase peroxides with alcohols (12).

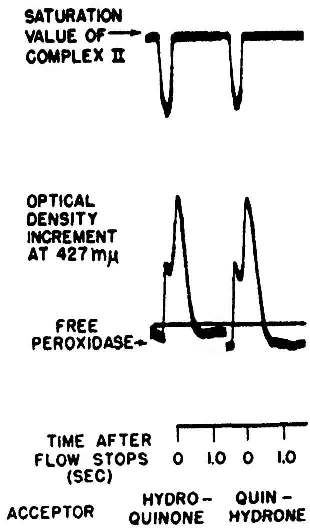

Fig. 2. A comparison of the reaction kinetics of Complex II in the presence of equal molarities of hydroquinone made up from pure hydroquinone or from quinhydrone (so labeled in figure). Note that the spectroscopic cycles are nearly identical (Expt. 339).

The third method of testing the significance of the effect of oxidation-reduction potentials is to employ different ratios of the oxidant and reductant in a system such as hydroquinone-quinone. Fig. 2 illustrates the reaction kinetics of Complex II in the presence of hydroquinone solutions of the same molarity. One solution is, however, made up from quinhydrone. The reaction kinetics of Complex II are identical to within the experimental error. Apparently a large amount of the oxidant (quinone) in this system does not slow the reaction of the reductant (hydroquinone) with Complex II.

Of these 3 tests, it is probable that only the first is significant. In the second test, the oxidation-reduction potential of the equilibrium,

$$AH_2 \rightleftharpoons A + 2H^+ + 2e,$$

is indeed affected by pH. But the reactions occurring here are represented above by Eqs. 1–3 and do not involve hydrogen ions. And in the third test, the concentration of the oxidant required to decrease the forward velocity of Eq. 3 is probably so high that spectrophotometric measurements of the kinetics of Complex II would be obscured.

The first test does give clear-cut results and shows that the rates of these oxidation reactions are rather remotely related to the oxidation-reduction potentials of the acceptors. It is possible, however, that these oxidation-reduction potentials do not correspond to those of the primary reaction of Complex II and the acceptor molecule (see also Michaelis (13)).

Conclusions

1. The speed of the reaction of Complex II with a group of acceptors chosen to represent the general types that peroxidase-peroxides oxidize is found to be only slightly affected by pH in the region 3.5 to 6.7. In two cases where significant changes were noted, corresponding changes in the nature of the acceptor or the reaction product probably occurred.

This lack of pH effect suggests that the reaction of Complex II and the acceptor molecule follows the general equation:

$$\text{Per OOH} - \text{II} + AH_2 \xrightarrow{k_4} \text{Per OH} + A + H_2O.$$

2. Under the conditions of these experiments, in which strong peroxidase is used, the rate of production of colored oxidation products of pyrogallol definitely lags the rate of disappearance of H_2O_2 and the measurement of peroxidase activity in terms of the overall reaction involves gross errors.

3. The value of k_4 for the reaction of Complex II and pyrogallol corresponds to a value of P.Z. = 1500.

4. The rate of disappearance of H_2O_2, as determined from the kinetics of Complex II, agrees closely with measurements of the rate of formation of tetraguaiacol (pH 3.5–6.7) and malachite green (pH 4.3–4.7).

5. The correlation between the oxidation-reduction potential of a number of different acceptors with the values of the velocity constant for their reaction with Complex II is slight. The correlation with changes in oxidation-reduction potential caused by changes in pH is not found. It is possible that these oxidation-reduction potentials do not correspond to the free energy change of the reaction that actually occurs between Complex II and the acceptor molecule.

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The Inactivation of Insulin by Tissue Extracts. III. The Effect of Force-Fed Diets on the Insulinase Activity of Rat Liver Extracts [1]

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Introduction

When adult rats are force-fed a high carbohydrate diet in progressively greater amounts, they become obese and ultimately exceed their tolerance for carbohydrates as manifested by the development of hyperglycemia and glycosuria (1). Further, the administration of insulin does not appreciably delay the onset nor reduce the severity of the glycosuria of such force-fed rats (2). Since the quantities of insulin which such rats withstand are relatively enormous, it would appear that force-feeding carbohydrate results in a relative insulin insensitivity, *i.e.*, insulin resistance.

The demonstration of an insulin-inactivating enzyme system (insulinase) in rat tissues (3) and the rapidity with which the activity of this system is reduced in the liver of rats by fasting and restored by refeeding (4) raised the question as to whether the effect of force-feeding of carbohydrate could be due to some change in the insulinase activity of the liver and a consequent increase in the rate of insulin destruction *in vivo*. For such reasons it became pertinent to examine the effect of the force-feeding of various diets on the insulinase activity of the liver.

Methods

Adult, male Sprague-Dawley rats weighing from 180 to 323 g. in body weight and averaging 237 ± 36.39 g.[3] were divided into three groups, each of which received a different one of the following diets: (a) A normal stock laboratory diet consisting of

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[2] With the technical assistance of Gladys Perisutti and Susan Bucher.

[3] Mean ± standard deviation.

Rockland Rat Diet ingested *ad lib.*; (b) a high carbohydrate diet administered by stomach tube; and (c) a high fat diet administered by stomach tube. The high carbohydrate and high fat diets were semifluid in consistency and 75% of their total caloric values were supplied in the form of carbohydrate and fat respectively. The compositions[4] of these diets were those specified by Ingle (5). The stock laboratory diet supplied 58% of its calories in the form of carbohydrate, 30% by protein, and 12% by fat. Sixteen rats received the stock diet, 7 rats the high carbohydrate diet, and 9 rats the high fat diet. The high carbohydrate and high fat diets were administered twice daily by stomach tube in accordance with the tube-feeding technique of Ingle (1). The tube-fed rats received gradually increasing quantities of their respective diets during a 5 day period of adaptation, and by the 5th day their daily intake was adjusted to a level of 26 ml./300 g. of body weight. Thereafter the amounts of high-fat and high-carbohydrate diets were increased by daily increments of 1 ml. This schedule of feeding was continued in this manner for either 1 week past the end of the adaptation period or, if they had failed to regain their initial weight during this latter period, for 1 week subsequent to the day on which they had regained their initial body weight. This procedure was adopted in order to keep the factor of weight change relatively constant. At the end of the feeding period, the rats were sacrificed by a blow on the head and their livers removed for assay of the insulinase activity. The animals fed the stock laboratory diet *ad lib.* for the same period of time served as controls for the values obtained in the groups of animals force-fed the high-carbohydrate and high-fat diets.

Each rat liver was homogenized with three volumes of ice-cold water in a Waring Blendor for 5 min. This homogenate was centrifuged at high speed and the resultant supernatant material filtered through fine gauze, adjusted to pH 7.5 and used immediately for assay. Each milliliter of the extract so obtained was considered equivalent to 0.3 g. of fresh liver. For the assay, 1 ml. of a particular liver extract was incubated for 30 min. at 37°C. with 1 ml. of a solution of amorphous insulin[5] containing 12 units/ml. At the end of the incubation period, the amount of insulin destroyed was determined by assay of the amount remaining. This latter was estimated by its hypoglycemic effect in adult male rabbits weighing between 3 and 4 lb. The procedure used has been completely described in an earlier communication and the results of the insulinase assays were expressed in terms of the *pooled percentage* (3,4). A high pooled percentage is indicative of a high insulinase activity and a low pooled percentage indicates a low activity. Thus, although this method does not allow for the accurate determination of the absolute quantity of insulinase in an extract, it does afford a means for the comparison of the relative activity of liver extracts obtained from different animals.

In this manner replicate reaction mixtures, each containing aliquots of liver ex-

[4] These diets were said by Ingle to supply 85% of their total caloric values in the form of carbohydrate or fat. As calculated by us, our diets supplied the caloric intake as follows: High carbohydrate diet—75% carbohydrate, 5% fat, 20% protein; high fat diet—75% fat, 5% carbohydrate, 20% protein. The differences between our figures and those of Ingle may be due to the composition of the Cellu flour used in our diets which contained 28.3% available carbohydrate and 56% protein.

[5] We are indebted to the Eli Lilly and Co. for generous supplies of highly purified amorphous insulin containing 22.5 units/mg.

tracts prepared from the same rats, were incubated with insulin and injected into each of 5 rabbits for the actual assay. Insulinase assays were thus performed on each of the livers obtained from 16 control rats fed the stock laboratory diet, on each of the livers from the 7 rats force-fed the high-carbohydrate diet, and on each of the livers from the 9 rats force-fed the high-fat diet. Since 5 rabbits were used for each assay, the 32 assays required the use of a total of 160 rabbits.

Results

For one reason or other, all of the rabbits failed to survive the scheduled injections. In order to avoid further complications, the mean values for the insulinase activities of each group of livers were calculated from the results of the determinations in the first four surviving rabbits of each group of five employed in these assays. The average insulinase values obtained in this manner (expressed in terms of the pooled percentages) for the livers of the high-fat force-fed rats and the control rats were essentially the same (Table I). The average value for the livers obtained from the high-carbohydrate force-fed rats was somewhat higher than those of the other two groups of animals, this value being 159 as contrasted to the values of 138 and 145 obtained in the control and high-fat force-fed groups. Statistical analysis of these results by the method of analysis of variance of data with two criteria of classification, one being a subclassification of the other (6), revealed, however, that these differences were not significant. This type of analysis disclosed that the difference between dietary treatments was not significantly greater than the differences in values obtained within a single treatment. Thus, within a single group of rats, the variability of

TABLE I

Effect of Force-Fed Diets on Rat Liver Insulinase

Type of diet	Number of rats	Liver insulinase activity (Pooled percentage)	Significance of differences
Stock rations, *ad lib.*	16	138 ± 37.55[a]	
High-carbohydrate force-fed	7	159 ± 36.31	Not significant ($p > 0.20$)
High-fat force-fed	9	145 ± 46.83	

[a] Insulinase activities expressed in terms of mean $\pm$ standard deviation.

results was too great to attach significance to the difference in pooled percentage values obtained between the three groups of rats.

Subsequently, it was discovered that the major factor responsible for the great degree of variability in the assay results was the change in the insulin sensitivity of some of the rabbits employed for assay purposes, a change which had been induced in these particular rabbits by subjecting them to previous intravenous injections of rat liver extracts (7). The principal source of variance within treatments was found to exist in the variance of the results obtained between the different groups of four rabbits used for replicate assays within the same dietary treatment group.

TABLE II

Effect of Force-Fed Diets on Rats

Type of diet	Number of rats	Gain in body weight	Liver weight	Liver weight / Body weight	Nitrogen content of liver extracts	Fasting blood sugar
		g.	*g.*		*Per cent*	*mg.-%*
Stock rations, *ad lib.*	16	18 ±16.32	7.4 ±1.09	0.030 ±0.005	0.89 ±0.035	79 ±14.85
High-carbohydrate force-fed	7	42 ±20.19	12.3 ±1.94	0.041 ±0.004	0.69 ±0.046	113 ±51.60
High-fat force-fed	9	38 ±12.73	10.0 ±1.62	0.038 ±0.012	0.75 ±0.059	108 ±24.01
Significance of difference		Highly significant ($p < 0.01$)	Highly significant ($p < 0.001$)	Significant ($0.05 > p > 0.01$)	Highly significant ($p < 0.001$)	Significant ($0.05 > p > 0.01$)

All values stated in terms of mean ± standard deviation.

Although the forced feeding of high-carbohydrate and high-fat diets did not result in any statistically significant changes in insulinase activity per gram of liver, certain other effects associated with differences in the dietary intake were noted and are illustrated in Table II. Rats force-fed with either the high-carbohydrate or high-fat diet gained more weight than the control rats allowed to feed *ad lib.* on the balanced stock diet; these differences in weight gains were statistically significant as shown by an analysis of variance. The livers of the high-fat force-fed rats were significantly heavier than those of the controls, and in turn the heaviest livers were those obtained from the high-carbohydrate force-fed rats; the liver weights of the carbohydrate force-fed rats were significantly greater than those of the fat force-fed group. The livers of the fat and carbohydrate force-fed rats were distinctly paler than

those of the control rats. The percentage nitrogen contents of the liver extracts from the high-fat force-fed rats were significantly less than those of the control rats, and in turn the percentage N content of the extracts from the high carbohydrate force-fed rats was significantly less than that from the fat force-fed group. As might be expected from the inverse ratio between liver weights and nitrogen contents of the extracts the quantity of nitrogen in the entire volume of extract obtained from the total liver mass did not show these same differences. This value was highest in the high-carbohydrate force-fed rats and lowest in the rats allowed to feed on the stock rations. The fasting blood sugar levels of the fat and carbohydrate force-fed rats were significantly higher than those of the control rats.

Discussion

The changes in insulin sensitivity induced as the result of previous injections of liver extracts (7) produced a greater variability within the rabbits injected with reaction mixtures obtained from the livers of the same group of rats than the variability found to exist between rabbits injected with reaction mixtures prepared from the three different groups of rats. As a result, it was not possible to attach any significance to such differences as were observed in the mean insulinase values for the three groups of rats. It is, of course, possible that a real difference in insulinase values in the livers of the three groups of rats might have been concealed by this type of variability. However, a series of preliminary experiments, designed to circumvent this difficulty, failed to indicate any real effect of force-feeding on liver insulinase activity. It would appear from the data that force-feeding, as practiced in these experiments, produced no significant changes in the activities of insulinase per gram of rat liver.

It will be recalled that the insulinase activities compare the insulinase contents per unit weight of liver. Since the force-fed diets resulted in the production of substantially larger livers than those found in the rats fed *ad lib.*, it would appear permissible to conclude that the force-fed rats contain more total liver insulinase than the rats fed with the stock diet. Furthermore, since the ratio of liver weight/body weight is highest in the rats force-fed carbohydrate, it would appear plausible that such rats contain more of this system per unit body weight than do the other two groups of rats. Whether or not such considerations may be useful in explaining the appearance of some of the symptoms of

diabetes and the apparent insulin resistance exhibited by rats force-fed a high-carbohydrate diet cannot be determined from the data now available.

Some types of insulin resistance, or some cases exhibiting an increase in insulin requirements, may be caused by a more rapid rate of insulin destruction within the body. It has been definitely established that insulin undergoes a rather rapid destruction subsequent to its injection (8). Further, insulin injected into the portal system is less effective than when injected peripherally (9). It is conceivable that this latter fact may be explained by the more rapid rate of destruction of insulin in the liver, which has been found to contain a higher content of insulinase than other tissues (3). In other words, since insulin injected directly into the portal system traverses the hepatic circulation before it can produce any effect on the peripheral tissues, the amount of insulin arriving at and acting on the latter tissues might be expected to be smaller when injected into the portal system than when an equivalent amount is administered peripherally. The proof of this hypothesis must await further investigation of the influence of alterations in liver insulinase content on insulin requirements.

Summary and Conclusions

1. Groups of rats were force-fed a high-carbohydrate and a high-fat diet. Another group of rats was fed *ad lib.* with the stock laboratory diet. Extracts were prepared from the livers of each of the rats from the three groups and these extracts were used for the determination of the effects of the three diets on the liver insulinase activity.

2. The three diets were found to produce no significant changes in the insulinase activity per unit weight of liver but significant changes were produced in the weights and other characteristics of the animals.

3. It was concluded that the force-fed diets may have produced an increase in the amount of liver insulinase per unit body weight.

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A Novel Synthesis of the Peptide Bond [1]

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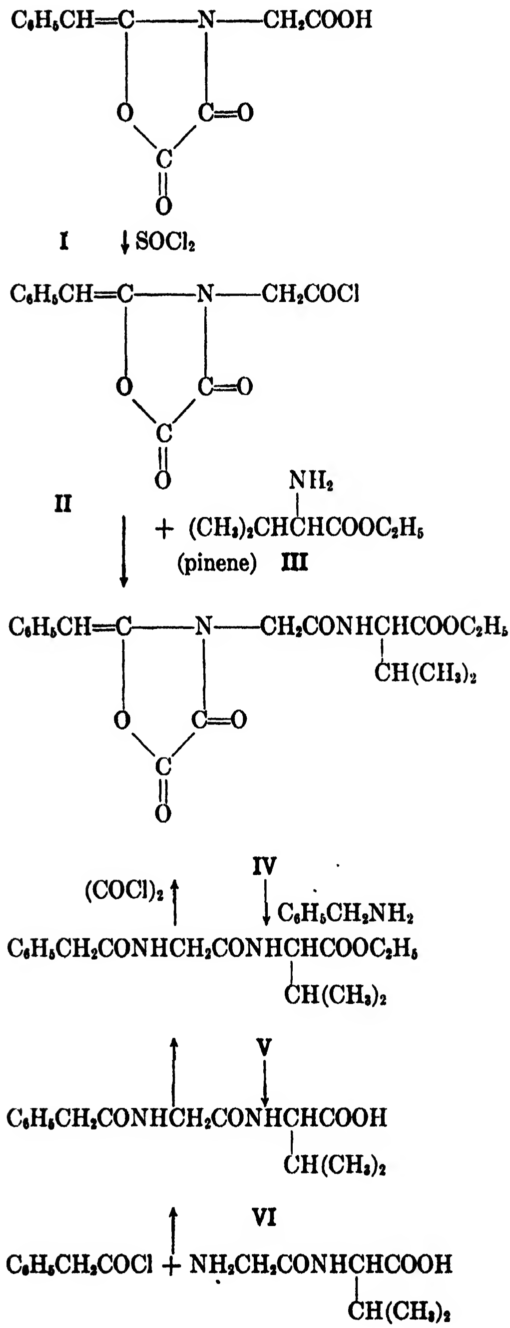
Introduction

In the course of certain studies of compounds related to pencillin it became of interest to attempt to mask an amide group with an easily removable substituent and to carry out further reactions in the presence of the protective grouping. The reaction of oxalyl chloride with the enol form of an amide leads (1,2) to a 4,5-diketoöxazolidine derivative which admirably serves the purpose of covering both the remaining hydrogen on the nitrogen of the substituted amide and also that of the enol form of the carbonyl group. The diketoöxazolidine derivative may be subsequently cleaved by the action of mild reagents. For instance, the action of benzylamine in dry ether, in which *sym*-dibenzyloxamide is insoluble, results (2) in the regeneration of the original amide in quantitative yield. Under these conditions the peptide linkage and the ester linkage are not affected.

In the case of the [2-benzal-3(4,5-diketoöxazolidine)]-acetic acid (I) it was easily possible to prepare the corresponding acid chloride (II), by treatment with thionyl chloride. Because of the extreme sensitivity of the oxazolidinedione to the action of aqueous acid or alkaline reagents, all usual techniques for the condensation of an acid chloride and an amine were unsuccessful. However, it was found that advantage could be taken of the facile addition of hydrogen chloride to pinene to absorb, in anhydrous solvents, the hydrogen chloride which is produced in the reaction between the acid chloride and an amine.

The condensation of [2-benzal-3(4,5-diketoöxazolidine)]-acetyl chloride (II) and valine ethyl ester (III) to yield *N*-[2-benzal-3(4,5-

[1] The work described in this paper was carried out under contract OEMcmr-411 between the Office of Scientific Research and Development and Cornell University Medical College and is described in Progress Reports to the Committee on Medical Research, January 1, 1944 to December 31, 1945.


I
SOCl₂
II
+ (CH₃)₂CHCHCOOC₂H₅
NH₂
(pinene) III
IV
(COCl)₂
C₆H₅CH₂NH₂
V
VI

diketoöxazolidine)]-acetyl-DL-valine ethyl ester, (IV), was accomplished in nearly quantitative yield. The cleavage of the diketoöxazolidine ring with benzylamine in ether led to *N*-(phenylacetylglycyl)-DL-valine ethyl ester, (V), which was in turn saponified to the acid (VI).

The phenylacetylglycylvaline and its ester were shown to be identical with samples synthesized by classical methods. In addition, the treatment of the authentic *N*-(*N*-phenylacetyglycyl)-DL-valine ethyl ester with oxalyl chloride, in ether, at room temperatue, yielded a diketooxazolidine derivative (IV), identical with that obtained by the procedure described.

Experimental

[2-Benzal-3(4,5-diketoöxazolidine)]-acetic Acid (I)

A suspension of 8 g. of phenaceturic acid in 450 ml. of dry ether was treated with 8 ml. of oxalyl chloride, and the mixture was shaken occasionally. After 8 hr., the starting material had dissolved and precipitation of yellow crystals had begun. After the mixture had remained at room temperature for 16 hr., 6.5 g. of yellow crystalline product was collected and washed with warm benzene. This represented 64% of the theoretical yield. The yellow crystals possessed a melting point of 221°.[2]

Anal. $C_{12}H_9O_5N$ (247.2). Calcd.: N, 5.67. Found: N 6.14.

Methyl-[2-benzal-3(4,5-diketoöxazolidine)]-acetate

A few milligrams [2-benzal-3(4,5-diketoöxazolidine)]-acetic acid (I) were treated with excess ethereal diazomethane. This resulted in solution of the starting material and simultaneous precipitation of yellow needles of the methyl ester, m.p. 186–187°. After recrystallization from hot benzene, the ester melted at 187–188° (2). The absorption spectrum, determined on a 0.01 mg./ml. solution in anhydrous ether, showed maxima at 3300 to 3400 A of E_M 13,800 and at 2450 A of E_M 16,700.

Methyl-N-ethoxalylphenaceturate

A sample of methyl-[2-benzal-3(4,5-diketoöxazolidine)]-acetate was warmed in 1 ml. of ethanol containing a trace of pyridine. When this solution was cooled and diluted with water, a crystalline product separated which was collected and washed with aqueous ethanol. The white plates thus obtained possessed a melting point of 95–96° (2).

[2-Benzal-3(4,5-diketothiazolidine)]-acetic Acid

A suspension of 1 g. of *N*-(phenylthioacetyl)-glycine in 45 ml. of ether was treated with 1 ml. of oxalyl chloride. There was evidence of immediate reaction and precipitation of a yellow crystalline product. After 4 hr., the product was collected. The yellow crystals amounted to 800 mg. (62% of the theoretical yield), m.p. 203–205°.

[2] All melting points were taken on a heated microscope stage.

The product was insoluble in hot benzene, chloroform, and hexane. It was washed with several portions of ether and then with chloroform.

Anal. $C_{12}H_9O_4NS$. (263.3). Calcd.: C, 54.73; H, 3.45; N, 5.33; S, 12.18. Found: C, 55.01; H, 3.72; N, 5.38; S, 12.76.

[2-Benzal-3(4,5-diketoöxazolidine)]-acetyl Chloride (II)

A suspension of 1 g. of [2-benzal-3(4,5-diketoöxazolidine)]-acetic acid (I) in 20 ml. of benzene and 5 ml. of thionyl chloride was heated to 60°. No reaction was apparent until a drop of pyridine was added. After it had been kept at 60–80° for 2 hr., the solution was quickly filtered and cooled. The yellow needles which crystallized amounted to 760 mg., representing 71% of the theoretical yield, m.p. 184–186°, with decomposition. The ultraviolet absorption spectrum of a 0.01 mg./ml. solution of this material in anhydrous ether possessed maxima at 3350 A of E_M 11,700 and at 2425 A of E_M 15,800.

Anal. $C_{12}H_8O_4NCl$. (265.5). Calcd.: Cl, 13.39. Found: Cl, 13.64.

The addition of a few milligrams of the acid chloride (II) to cold methanol resulted in rapid solution, followed by crystallization of yellow needles. These were collected immediately and dried on filter paper. They possessed a melting point of 188–189° and admixture with the parent acid chloride lowered the melting point to 160–165°. Admixture of the ester, m.p. 188–189°, with an authentic sample of methyl [2-benzal-3(4,5-diketoöxazolidine)]-acetate did not depress the melting point.

N-*[2-Benzal-3(4,5-diketoöxazolidine)]-acetyl-*DL*-valine Ethyl Ester (IV)*

(*a*) A suspension of 1.0 g. of [2-benzal-3(4,5-diketoöxazolidine)]-acetyl chloride (II) in 15 ml. of carbon tetrachloride, 15 ml. of ether, and 25 ml. of pinene was stirred vigorously and 0.60 g. of DL-valine ethyl ester (III) in 10 ml. of carbon tetrachloride was added. After the reaction mixture had been stirred for 36 hr. at room temperature, the product was collected and recrystallized from 50 ml. of benzene. The yellow crystals weighed 1.25 g., which represents 88% of the theoretical yield, and melted at 168–169°.

(*b*) A solution of 500 mg. of the acid chloride, II, in 75 ml. of benzene and 20 ml. of pinene was warmed to 75°, and 300 mg. of DL-valine ethyl ester in 10 ml. of benzene was added. The solution was allowed to stand at room temperature for 3 hr. and 630 mg. of crystalline material (89% of the theoretical yield), m.p. 168–170°, was then collected. After 2 recrystallizations from 20 ml. of hot benzene the crystals melted at 169–170°.

Anal. $C_{19}H_{22}O_6N_2$. (374.4). Calcd.: C, 60.95; H, 5.92; N, 7.49. Found: C, 61.59; H, 6.13; N, 7.72.

The ultraviolet absorption spectrum of this product, determined on a 0.01 mg./ml. solution of the compound in ether, possessed maxima at 3350 A of E_M 11,300 and at 2450 A of E_M 12,800.

N-*(*N-*Phenylacetylglycyl)-*DL*-valine Ethyl Ester (V) from* N-*([2-Benzal-3 (4,5-diketoöxazolidine)]acetyl)*DL*-valine Ethyl Ester (IV)*

To a solution of 374 mg. of *N*-([2-benzal-3(4,5-diketoöxazolidine)]-acetyl)DL-valine ethyl ester (IV) in 20 ml. of ether was added 220 mg. of benzylamine. The

yellow color of the diketoöxazolidine was rapidly discharged and a white precipitate began to form in a few minutes. After 90 min. the *sym*-dibenzyloxamide, m. p. 221–223°, was collected. It amounted to 255 mg., representing a quantitative yield. The ethereal mother liquors were diluted with 2 volumes of hexane and cooled. The white plates which separated, m. p. 94–95°, amounted to 237 mg., and represented 74% of the theoretical yield. The latter compound was recrystallized twice by solution in 20 ml. of boiling ether and addition of 2 volumes of hexane to the cooled solution. The recrystallized material melted at 95–96°. A mixture of this product with an authentic sample of *N*-(*N*-phenylacetylglycyl)-DL-valine ethyl ester (V), described below, showed no depression of the melting point.

Anal. $C_{17}H_{24}O_4N_2$ (320.4). Calcd.: C, 63.71; H, 7.51; N, 8.94. Found: C, 64.20; H, 7.58; N, 9.01.

Preparation of N-(N-*Phenylacetylglycyl*)-DL-*valine* (*VI*) *from the Ester* (*V*)

A sample, 64 mg., of the *N*-(*N*-phenylacetylglycyl)-DL-valine ethyl ester, m. p. 95–96° (V), was dissolved in 0.5 ml. of ice cold ethanol and 0.2 ml. of 1 *N* sodium hydroxide was added. After it had been kept in an ice bath for 45 min., the solution was filtered to remove a small amount of insoluble material. The solution was acidified with 2 ml. of 0.1 *N* hydrochloric acid and cooled. The crystals which were obtained possessed a melting point of 141–142° and admixture with an authentic sample of *N*-(*N*-phenylacetylglycyl)-DL-valine, described below, showed no depression of the melting point. This product amounted to 32 mg., representing 55% of the theoretical quantity.

N-(N-*Phenylacetylglycyl*)-DL-*valine*

A solution of 1.74 g. of glycyl-DL-valine, [m. p. 240° (3)] in 10 ml. of 1 *N* sodium hydroxide was treated at 0° with alternate portions of 1.8 g. of phenylacetyl chloride and 16 ml. of 1 *N* sodium hydroxide. After 4 hr. the solution was acidified with 26 ml. of 1 *N* sulfuric acid and the precipitated material was recrystallized from 80% ethanol. The product crystallized as plates, m. p. 142–143°, and weighed 1.6 g., representing 55% of the theoretical yield.

Anal. $C_{15}H_{20}O_4N_2$ (292.4). Calcd.: N, 9.61. Found: N, 9.90.

Esterification of N-(N-*Phenylacetylglycyl*)-DL-*valine*

A 150 mg. sample of the compound was dissolved in 5 ml. of saturated ethanolic hydrogen chloride and this was evaporated to dryness below 40°. This procedure was repeated twice. The residual oil was dissolved in 10 ml. of ether, filtered from a small amount of high-melting solid material, and precipitated by hexane. Several such re-precipitations yielded an oil which was layered with 5 ml. of ether and cooled at 5°. After 2 days appreciable crystallization had occurred and the product was collected and washed with ether. Recrystallization from a small volume of ether yielded 50 mg. of prisms, m. p. 94–95°, of *N*-(*N*-phenylacetylglycyl)-DL-valine ethyl ester, (V).

N([*2-Benzal-3*(*4,5-diketoöxazolidine*)]-*acetyl*)-DL-*valine Ethyl Ester* (*IV*) *from* N-(N-*Phenylacetylglycyl*)-DL-*valine Ethyl Ester* (*V*)

A solution of 20 mg. of V in 5 ml. of ether was treated with a drop of oxalyl chloride. The development of a yellow color and the appearance of a precipitate occurred almost

immediately. The crude product melted at *ca.* 150°, but after 2 recrystallizations from benzene it melted at 167–170° and a mixture with *N*-[2-benzal-3(4,5-diketoöxazolidine) acetyl]-DL-valine ethyl ester showed no depression of the melting point.

Treatment of N-([*2-Benzal-3(4,5-diketoöxazolidine)*]*-acetyl)-*DL*-valine Ethyl Ester (IV) with Excess Oxalyl Chloride*

Although a suspension of this ester in ether did not react appreciably with oxalyl chloride, it was thought that reaction might occur in solution in warm benzene. A solution of 25 mg. of IV in 3 ml. of benzene at 80° was treated with a drop of oxalyl chloride. After being kept at 80° for 30 min., the reaction mixture was cooled. The product, which crystallized, melted at *ca.* 150°, and after recrystallization from benzene it melted at 167–170°. This material was identical with the starting ester. Several milligrams of material remained after evaporation of the benzene mother liquors. This started to melt at about 125° and this melting point was further depressed by admixture with IV. This behavoir suggests that some further reaction of oxalylchloride with the second, sterically hindered, amide group may have occurred.

Acknowledgment

The author wishes to express his appreciation to Prof. Vincent du Vigneaud for his continued advice and counsel and to Mr. Roscoe C. Funk, Jr., for the microanalyses.

Summary

In the course of a synthesis of a peptide linkage, pinene was used to absorb the hydrogen chloride formed in the condensation of an acid chloride and an amine.

Several derivatives were prepared from the 4,5-diketoöxazolidine derived from phenylacetylglycine and a 4,5-diketothiazolidine derivative is reported.

A synthesis of phenylacetylglycylvaline and derivatives thereof is described.

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The Nutritional Requirements of a Bacitracin-Producing Strain of *Bacillus subtilis* [1]

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Introduction

Bacitracin, an antibiotic highly effective against hemolytic streptococci, produced by a strain of *Bacillus subtilis* was first described by Johnson *et al.* (1). The antibiotic was produced in surface culture on a synthetic medium. Attempts to produce the antibiotic by submerged aerated cultures were unsuccessful (1,2). This paper presents data on the production of bacitracin by submerged aerated cultures in synthetic mediums and observations on the nutritional requirements of the organism.

Standard Conditions

Medium

In these experiments a modification of the medium employed in the production of bacitracin and tyrothricin was used (2,3) as follows:

L-Glutamic acid	10.0 g.
Glucose	5.0 g.
Citric acid	1.0 g.
K_2HPO_4	0.5 g.
KH_2PO_4	0.5 g.
$MgSO_4 \cdot 7H_2O$	0.2 g.
$MnSO_4 \cdot 4H_2O$	0.01 g.
$FeSO_4 \cdot 7H_2O$	0.01 g.

Distilled, demineralized H_2O to 1000 ml.

The medium, with glucose omitted, was adjusted to pH 6.8–7.0 with NaOH, and then sterilized for 20 min. at 15 lb. pressure. After sterilization 1 ml. of a 20% sterile

[1] The work described in this paper was conducted with the helpful advice of Dr. S. A. Waksman in partial fulfillment of requirements for the Ph.D. degree in the Department of Microbiology, Rutgers University.

glucose solution was aseptically added to each flask since autoclaving the glucose in the presence of phosphate resulted in caramelization. As with surface cultures, (2) excessive carmelization proved toxic to the growth of the organism, which resulted in poor bacitracin titers.

All the glassware used was cleaned with dichromate-acid solution and thoroughly rinsed with distilled water, which had previously been passed through an ion-exchange resin column. All water used in the experimental work was treated similarly. All chemicals used were reagent grade Merck.

Inoculum

Cultures used for the preparation of inoculum were maintained on Difco nutrient-agar medium.[3] Inoculated slants were incubated at 37°C. for 24–48 hr. and then stored at icebox temperatures until used.

Each flask was inoculated with 1 ml. of a spore suspension derived from an agar slant culture and containing approximately 500×10^{8} spores/ml. When 48-hr. submerged growth was used as inoculum there was a marked increase in the rate of growth and bacitracin production. While the lag phase was thus decreased by 24–48 hr., there was no significant change in the total cell concentration or bacitracin produced.

Incubation

The inoculated flasks were incubated at 28°C., on a shaking machine imparting a rotary motion of 220 r.p.m.

Determinations

Bacitracin titer was determined by a cup assay procedure developed in this laboratory.

Cell weight was determined turbidimetrically.

Glucose was determined by a modification of the Somogyi method.

pH was determined potentiometrically.

In the following experiments various constituents of the medium and several environmental factors were varied, individually, in order to determine the optimal fermentation conditions for bacitracin production.

Fermentation Scheme

The course of fermentation in bacitracin production is presented in Fig. 1. Growth commenced between the first and second days, reached a peak on the third day and then decreased on the fourth day to approximately one-half of maximum.

Bacitracin titers followed the growth curve fairly well. Maximum

[3] The culture was kindly supplied by Dr. F. L. Meleney, Department of Surgery, College of Physicians and Surgeons, Columbia University, New York.

titers were obtained on the third to fourth days. After the fifth day there was a decrease in bacitracin titer.

Sugar consumption was rapid once growth was initiated, and complete on the third day of incubation.

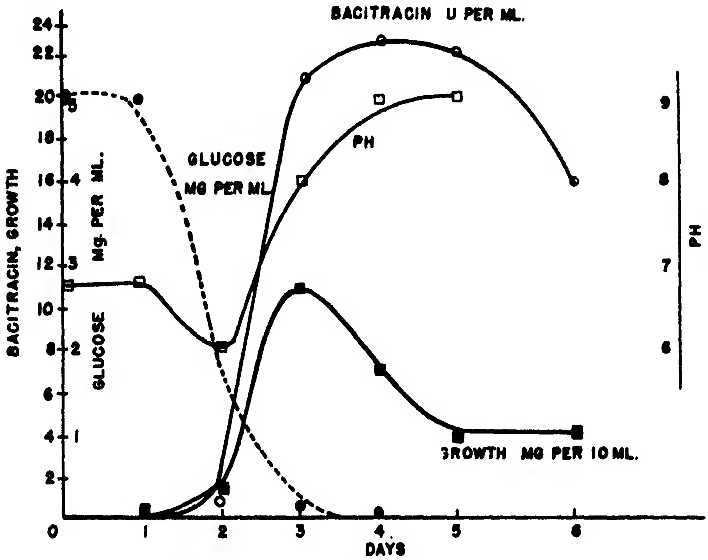

Fig. 1. General fermentation scheme.

The reaction of the culture dropped to pH 6.0 with rapid utilization of the carbohydrate and then rose rapidly with depletion of sugar and increased deamination, reaching a value of 9.0–9.5 on the fourth day.

General Factors

Supplementation with Citrate

The use of phosphate in the presence of metallic ions leads to the formation of highly insoluble salts making the metal ions unavailable to the microorganism. The addition of organic acids, such as citrate, leads to the formation of soluble coordinate complexes with the metal ions thus making them available to the microorganism (4,5,6). The addition of 0.1% citric acid eliminated the turbidity previously encountered in the medium and made for better reproducibility among replicate flasks. The metals bound as citrate complexes are unavailable to some microorganisms (5,7). Such was not the case with *Bacillus subtilis*, since there was no metal deficiency in the presence of citrate.

Effect of Aeration

The effect of aeration on bacitracin production was investigated by varying the quantities of medium in 250 ml. Erlenmeyer flasks while maintaining the other conditions constant. Flasks containing 20, 40, and 60 ml. of medium showed more rapid growth and bacitracin production than did flasks containing 80 and 100 ml. of medium (Table I). Variations in medium volume affected only the rate of growth and antibiotic production, but had no significant effect on maximum cell and bacitracin yields.

TABLE I

Effect of Aeration on Bacitracin Production

Volume[a] (ml.)	Growth (mg./l.)			Bacitracin titer (units/ml.)			pH		
	Days			Days			Days		
	2	3	4	2	3	4	2	3	4
20	750	840	670	8.7	22.2	21.6	7.3	9.1	9.4
40	280	1140	800	<2.0	22.2	24.2	6.0	8.5	9.2
60	174	1170	800	<2.0	20.8	26.0	6.0	8.2	9.1
80	70	780	1010	<2.0	13.4	23.4	6.3	7.7	8.9
100	45	485	1145	<2.0	10.5	25.7	6.7	7.5	8.5

[a] Volume of broth/250 ml. Erlenmeyer flask.

Temperature of Incubation

While the maximum bacitracin titers produced were the same at 24, 28, and 37°C., when standard conditions were employed, the rate of bacitracin production varied directly with the incubation temperature. As shown in Table II, maximum titers were obtained at 1–2 days, 3–4 days and 4–5 days, respectively, at 37,28, and 24°C. It was also observed that loss of titer was more rapid at 37°C. than at 28°C. As much as 50% of the bacitracin was lost in 12–24 hr. after peak production at 37°C., while no significant loss was observed at 28°C. over a period of 24–48 hr. after peak production was obtained.

TABLE II

Effect of Temperature on Bacitracin Production

Temp., °C.	Growth (mg./l.)				Bacitracin titer (units/ml.)				pH			
	Days				Days				Days			
	2	3	4	5	2	3	4	5	2	3	4	5
24	8	85	480	700	<2.0	<2.0	9.8	20.0	6.4	6.2	7.8	8.5
28	108	810	745	385	<2.0	9.6	21.5	22.3	6.0	7.1	9.1	9.0
37	950	720	525	380	19.0	9.0	8.0	5.3	8.6	9.4	9.6	9.3

Supplementation with Growth Factors

While the addition of a mixture of growth factors[3] to the glutamate medium was without effect, under standard conditions, on the total cell or bacitracin yields, it did decrease the lag phase by 24-48 hr.

Initial pH of Medium

B. subtilis was incapable of growing at pH 4.5 under our standard experimental conditions (Table III). At pH 5.0 growth was very slow, maximum growth being reached on the seventh day. While growth occurred at approximately the same rate at pH levels of 5.4–8.3, 25% higher bacitracin titers were obtained at pH 7.4 and 8.3 than at 6.8.

TABLE III

Initial pH of Medium and Bacitracin Production

pH	Maximum cell concentration mg./l.	Maximum activity units/ml.
4.5	0.0[a]	0.0[a]
5.0	1020[a]	4.8[a]
5.4	820	12.1
6.1	950	19.7
6.5	1080	21.6
6.9	910	18.0
7.4	900	27.3
8.3	920	27.9

[a] After 7 days incubation. The other data were obtained on the third day of incubation.

[3] Adenine 10, guanine 10, uracil 10, riboflavin 0.2, pantothenic acid 0.2, thiamine 0.2, pyridoxamine 0.4, biotin 0.0002, *p*-aminobenzoic acid 0.04, nicotinic acid 0.2, and folic acid 0.002 μg./ml.

Mineral Requirements

The synthetic medium of Anker *et al.* (2) contained salts of Ca, Cu, Fe, Mg, Mn, Na, and K. Preliminary experiments showed Ca and Cu to be unnecessary for growth and bacitracin production. More than that, in several experiments it appeared that the Ca and Cu ions, while not affecting growth of the microorganism, did significantly decrease bacitracin titers. These salts have therefore been eliminated from the medium used in our experiments.

TABLE IV

Effect of K and Phosphate on Bacitracin Production

Phosphate concentration[a]	Potassium salt		Sodium salt	
	Maximum cell concentration	Maximum activity	Maximum cell concentration	Maximum activity
mg./ml.	*mg./l.*	*units/ml.*	*mg./l.*	*units/ml.*
0.0[b]	0.0	<2.0	0.0	<2.0
0.1	140	4.0	70	<2.0
0.5	800	19.0	160	<2.0
1.0	700	19.0	160	<2.0
1.5	820	23.8	180	<2.0
2.0	800	22.9	200	<2.0
4.0	780	16.1	180	<2.0
8.0	800	16.1	180	<2.0
10.0	800	18.1	200	<2.0
20.0	720	18.9	200	<2.0
2.0[b]	800	21.0	850	22.2

[a] Mixture of mono-and dibasic phosphates at pH 7.0.
[b] KCl added at 1.0 mg./ml.

The addition of NaCl was found unnecessary since neutralization of glutamic acid with NaOH insured sufficient Na; Cl did not appear to be important for bacitracin production.

The addition of phosphate was found essential. The substitution of Na_2HPO_4 for K_2HPO_4 markedly decreased growth of the organism so that very little, if any, cell substance was obtained (Table IV). These experiments proved the essentiality of K ion for the growth of *B. subtilis*. The phosphate and K results are in agreement with those reported by Feeney *et al.* (6) for the production of subtilin.

Magnesium

Experimental evidence was obtained which showed Mg to be essential for the growth of *B. subtilis*. The relationship between Mg concentration and growth and bacitracin titer can be seen in Fig. 2. In the medium used, 2 p.p.m. Mg appeared to be optimal for both growth and

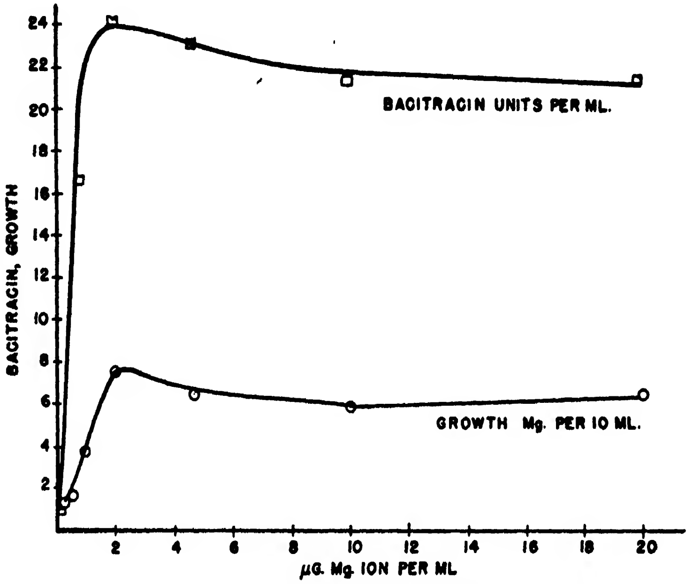

FIG. 2. Mg ion concentration and bacitracin production. Medium contains 2.0 μg./ml. Mn and 2.0μg./ml. Fe.

activity. In subtilin production, however, two optima of Mg were found, one for growth and one for activity (6).

Manganese

In recent years manganese has been reported to be essential in the production of bacillin (8) and subtilin (9). Experimental data have been obtained which show that Mn is also essential for antibiotic production by the bacitracin-producing strain of *B. subtilis*. It is evident from Table V that no significant growth occurred in the absence of Mn, except when high concentrations of Mg and Fe were present. The addition of as little as 0.01 μg/ml. of Mn produced almost maximal growth, but no bacitracin. At concentrations of 0.1–0.5 μg/ml. of Mn, maximal bacitracin titers were obtained. That this Mn effect is not due

TABLE V

Metal Concentration and Bacitracin Production

μg./ml.			Final pH	Maximum cell concentration	Maximum activity
Mn	Fe	Mg			
				mg./l.	*units/ml.*
0.0	2.5	20.0	8.6	280	0.0
0.01	2.5	20.0	9.3	725	0.0
0.03	2.5	20 0	9.4	580	5.5
0.05	2.5	20.0	9.4	830	10.5
0.07	2.5	20.0	9.4	900	12.9
0.1	2.5	20.0	9.4	1190	17.5
0.5	2.5	20.0	9.5	1010	20.8
1.0	2 5	20.0	9.5	1000	22.6
2.0	2.5	20.0	9.5	870	22.3
0.0	2.5	200.0	9.0	400	0.0
0.0	12.5	100.0	9.2	900	0.0
2.0	2.5	0.0	5.5	50	0.0
50.0	2.5	0.0	6.9	25	0.0

to impurities in the reagent salts used has been demonstrated by obtaining the same effect with electrolytically purified Mn metal.[4]

It was further observed that Mg at concentrations as high as 200 μg/ml. could not replace Mn for growth. Fifty times the optimal Mn concentration did not replace the growth-promoting property of Mg (Table V).

Iron

Iron has been shown to be essential in minute quantities for the growth of several microorganisms (10). In this study no attempt was made to purify the constituents of the medium with respect to Fe as has been done by others (6,10, 11).In several cases it was observed that added Fe, while not significantly affecting the growth of the organism, did enhance bacitracin titers in the optimal medium. In this respect Fe appeared to supplement the bacitracin-producing capacity of Mn, but was unable to replace Mn. However, Fe and Mg together did in

[4] Mn prepared electrolytically by the U. S. Bureau of Mines was obtained through the courtesy of Dr. S. H. Hutner, Haskin Laboratories, New York. The nitrate was formed by dissolving the metal in concentrated HNO_3, Merck reagent grade.

several experiments completely replace Mn with respect to growth (Table V). While maximal growth could be obtained in Mn-deficient mediums by increasing the Fe and Mg content, no bacitracin could be detected.

Other Metals

The addition of Zn, Mo, and Cr at concentrations of 2 and 10 μg/ml. had no effect on bacitracin titers or cell growth. While 2 μg/ml. of added Cu and Ni had no effect, 10 μg/ml. of these metals reduced bacitracin titers by approximately 20% without significantly changing the cell content of the medium.

Carbon and Energy Requirements

The bacitracin-producing strain of *B. subtilis* was unable to grow in a medium containing amino acids as a sole source of carbon and energy.

TABLE VI

Carbon Sources and Bacitracin Production

Carbon source added[a]	Maximum cell concentration *mg./l.*	Maximum activity *units/ml.*
0.1% Citrate alone	500	9.0
Xylose	1160	30.0
Ribose	670	26.6
Arabinose	1290	22.1
Rhamnose	1260	36.2
Glucose	1150	22.2
Mannose	1210	26.0
Galactose	1230	28.7
Levulose	1130	28.0
Maltose	740	24.7
Sucrose	1060	24.9
Trehalose	560	29.0
Melibiose$\cdot H_2O$	740	41.9
Cellobiose	680	24.4
Raffinose	800	42.0
Inulin	770	23.5
Dextrin	810	25.2
Sorbitol	960	25.4
Mannitol	960	25.2
Inositol	1080	24.9

[a] Carbon sources at 0.5% concentration. In mediums containing D-arabinose, sorbose, lactose, melezitose, salicin, adonitol, and dulcitol, no significant yields of bacitracin were obtained.

Glucose and many other carbohydrates[5] (Table VI) as well as organic acids and alcohols appeared to be readily utilized by this organism. The sodium salts of acetic, succinic, tartaric, and gluconic acids produced growth and bacitracin titers equivalent to glucose. Formate and ethyl oxalacetate did not support growth and in some cases appeared to be toxic, while lactate produced titers significantly higher than the control (Table VII).

TABLE VII

Utilization of Organic Acids

Carbon source added[a]	Maximum cell concentration *mg./l.*	Maximum activity *units/ml.*
0.1% Citrate alone	500	9.0
Glucose (control)	980	19.0
Formic	0.0	0.0
Acetic	670	17.8
Lactic	1190	35.4
Oxalic	990	7.8
Succinic	935	19.9
Malonic	770	10.4
Citric	1150	21.3
Tartaric	760	15.3
2-Ketoglutaric[b]	740	14.0
Oxalacetic[c]	20	0.0

[a] Acid was added as sodium salt at concentrations equivalent to carbon in 0.5% glucose.

[b] Added as Ca salt.

[c] Added as the sodium salt of the ethyl ester.

Nitrogen Requirements

Inorganic Nitrogen

B. subtilis was able to utilize ammonium and nitrate as sole nitrogen sources in the salts-dextrose medium. The growth and bacitracin titers in inorganic nitrogen mediums were, however, singificantly lower than in mediums containing organic nitrogen. While 700 mg./l. of cell substance was obtained in an $(NH_4)_2HPO_4$ medium, only 10 units/ml. of bacitracin was produced. That the pH of the medium was critical has been previously demonstrated. This point was further emphasized by the data obtained with ammonium salts. In a phosphate

[5] The carbohydrates were sterilized separately and added aseptically to the medium in order to prevent caramelization.

supplemented medium $(NH_4)_2HPO_4$ was superior to $(NH_4)_2SO_4$ with respect to growth and activity production. The low values with $(NH_4)_2SO_4$ can be attributed to the acidity produced when the ammonium was utilized.

Amino Acids

L-Glutamic acid, DL-aspartic acid, L-proline, and DL-alanine were utilized by the microorganism for growth and bacitracin production as sole nitrogen sources (Table VIII). Bacitracin titers of 25–40 units/ml. were obtained with the two dicarboxylic acids, while L-proline and DL-alanine yielded 15–20 units/ml. The following amino acids did not support growth when used as sole nitrogen sources: DL-threonine, DL-valine, glycine, DL-leucine, DL-isoleucine, DL-norleucine, DL-phenylalanine, β-alanine, L-lysine, L-hydroxyproline, DL-tryptophan, L-tryosine, DL-methionine, L-cystine, L-cysteine, and L-histidine.

TABLE VIII

Amino Acid Utilization and Inhibition

Amino acid	Glutamate medium[a]		Salts medium[b]	
	Cell conc.	Activity	Cell conc.	Activity
	mg./l.	*units/ml.*	*mg./l.*	*units/ml.*
DL-α-Alanine	1160	12.2	830	18.5
DL-Aspartic acid	1140	25.10	1020	28.0
L-Proline	1080	15.0	1160	18.0
DL-Leucine	680[c]	7.1	0.0	0.0
L-Cystine	480[c]	7.3	0.0	0.0
DL-Norleucine	1360	5.8	0.0	0.0
DL-Valine	630	8.5	0.0	0.0
DL-Tryptophan	0.0	0.0	0.0	0.0
Control	980	18.5	870	28.0

[a] Amino acid added at 0.5% concentration.
[b] Amino acid concentration equivalent to N in 1% glutamate.
[c] On fourth day of incubation.

Supplementation of the glutamic acid medium with DL-aspartic acid, α-alanine or L-proline was without significant effect on bacitracin titers. When the glutamic acid medium was supplemented with each of the non-utilizable amino acids at 0.5% concentration, no significant change in bacitracin titer was observed except in the case of the five following

amino acids. DL-leucine, DL-norleucine, DL-valine, and L-cystine reduced bacitracin titers by 50% or more. The addition of DL-tryptophan completely inhibited the growth of the microorganism (Table VIII).

SUMMARY

The factors influencing bacitracin production and the nutritional requirements of *B. subtilis* were investigated. The pH of the medium, degree of aeration, and temperature of incubation were found to affect bacitracin production.

The addition of Mg, Mn, Fe, K, and P was essential to growth and bacitracin production. Mn was found essential to bacitracin production.

Many carbohydrates, organic acids, and sugar alcohols were utilized as energy and carbon sources.

Inorganic nitrogen in the form of ammonium and nitrate, aspartic and glutamic acids, α-alanine and proline were utilized as sole nitrogen sources. The supplementation of the glutamate medium with DL-leucine, DL-norleucine, DL-valine, L-cystine, and DL-tryptophan resulted in a marked decrease in growth and bacitracin titer.

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The Effect of N-Substituted Amino Acids on the Growth of Lactobacilli [1]

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Introduction

In connection with a microbiological evaluation of amino acid analogues as possible metabolic antagonists a number of N-substituted amino acids have been prepared and made available to this laboratory by Dr. Emery M. Gal of the Department of Biochemistry, University of California, Berkeley. The details of the preparative work have recently been published (1).

Experimental

To investigate the competitive inhibition potencies of these compounds with lactobacilli the basal uniform medium formulated by Henderson and Snell (2) was employed. This was prepared so as to omit the one amino acid under study and divided into two portions. To the first portion the test amino acid was added to a final concentration of 5μg./ml. (all quantities of amino acids are reported in terms of the L-isomer). The other portion was left unsupplemented for use in the construction of a standard curve of reference. One milliliter of the appropriate basal medium was then added to each assay tube. The reference curve was prepared with the test amino acid in concentrations ranging from 0–20 μg. In most experiments the amino acid analogues were added to the 5-μg. supplemented tubes in increasing amounts and all tubes brought up to a final volume of 2 ml. after which plugging and sterilization (10 lb. for 10 min.) was carried out. In other experiments the tubes were prepared as described except that the analogues were sterilized by Seitz filtration and later added to the previously sterilized and cooled tubes. All tubes were then inoculated with one drop of a moderately turbid saline suspension from a washed 24-hr. broth culture of a lactobacillus and incubated for 72 hr. at 37°C. Growth was estimated at the expiration of this period by electrometric titration of the lactic acid produced using 0.02 N NaOH.

[1] This investigation was supported (in part) by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

Results and Discussion

Table I lists the test organisms employed, the test amino acids, the maximum quantities of the DL-*N*-substituted amino acids which were used and the effect of these latter compounds on the growth of the organisms as judged by acid production. The maximal quantities of the analogues which were tested were usually determined by a consideration of solubility since many proved to be difficultly soluble in the basal medium.

The use of Seitz-filtered samples was suggested by the observations on the stimulatory action of some of the *N*-substituted compounds since it was felt that these responses might be due to hydrolysis to the free amino acid as a result of autoclaving. Under these conditions most of the compounds found to be stimulatory after autoclaving now failed to influence acid production which was exactly equal to that in the control tubes. An exception was noted in the case of the *N*-substituted phenyl derivatives where inhibitory action was demonstrable after Seitz filtration although the autoclaved samples had been stimulatory.

In identification of these *N*-substituted amino acids by paper chromatography, Gal (3) reported that while these compounds responded with spot formation, they gave spots far below the intensity of their corresponding amino acids. Color intensity could be correlated with the rate of hydrolysis of the substituent, and the positive ninhydrin color was interpreted as due to a certain degree of hydrolysis which took place under the effects of the solvents and heating used in developing the chromatograms. These observations taken together with the failure in our experiments of a Seitz-filtered sample to increase acid production suggest that the stimulatory action which we have noted was an artifact induced by autoclaving. In the case of the *N*-substituted phenyl derivatives, it is suggested that their inhibitory activity, demonstrated in the filtered samples, was reversed by the presence of small amounts of phenylalanine due to hydrolysis in the autoclaved samples. This is supported by the observation that 1 to 10 μg. of phenylalanine added to a series of tubes each containing 500 μg. of a filtered preparation of the *N*-phenyl phenylalanine produced a growth curve only slightly depressed below the standard curve containing similar quantities of phenylalanine without inhibitor.

Experiments were also carried out with the *N*-substituted phenylalanine derivatives (excepting the toluyl compound which was not avail-

TABLE I

The Effect of N-Substituted Amino Acid Analogues on the Growth of Lactobacilli

Amino acid analogue and maximum quantity of DL compound used	Effect on acid production (Expressed as amount equivalent to 1 μg. of amino acid. S = stimulatory; I = inhibitory; O = no effect) Autoclaved	Seitz-filtered
Test organism—*Leuconostoc mesenteroides*, P-60		
Test amino acid—Valine (5 μg.)		
N-ethylvaline 2250 μg.	0	
N-ethylnorvaline 2250 μg.	I 2250	
N-isopropylvaline 2250 μg.	I 468 or 0 (see text)	
N-propylvaline 2250 μg.	0	
Test organism—*Lactobacillus arabinosus*		
Test amino acid—Leucine (5 μg.)		
N-phenylleucine 2250 μg.	S 274	I 1820
N-ethylleucine 2250 μg.	S 1250	0
N-isopropylleucine 2250 μg.	S 340	0
N-propylleucine 2250 μg.	S 178	0
Test organism—*Leuconostoc mesenteroides*, P-60		
Test amino acid—Phenylalanine (5 μg.)		
N-ethylphenylalanine 900 μg.	S 205	0
N-propylphenylalanine 900 μg.	S 100	0
N-isopropylphenyllanine 900 μg.	S 102	0
N-phenylphenylalanine 900 μg.	S 90	I 290
N-toluylphenylalanine 900 μg.	S 115	
Test organism—*Streptococcus fecalis*		
Test amino acid—Threonine (5 μg.)		
N-ethylaminobutyric acid 2250 μg.	0	
Test organism—*Leuconostoc mesenteroides*, P-60		
Test amino acid—Methionine (5 μg.)		
N-ethylaminobutyric acid 900 μg.	0	

able in sufficient supply) using an organism, *Lactobacillus lycopersici* (4005), which does not require an exogenous source of phenylalanine. A phenylalanine-free basal medium was used as before but this amino acid was not added. The addition of the *N*-substituted derivative up to

900 μg./tube effected no change in acid production over the unsupplemented controls. It was therefore concluded that these derivatives do not influence the synthesis of phenylalanine by this organism.

Summary

A number of *N*-substituted amino acids have been evaluated as possible antagonists to corresponding amino acids in the growth of lactobacilli. In the majority of cases either no effect or a stimulating effect was observed when the compounds were subjected to autoclaving incident to sterilization. The stimulatory effect was shown to be due probably to some hydrolysis to the free amino acid since Seitz-filtered samples produced no effect or, in the case of two phenyl substituted analogues, an inhibition of growth which was reversed by the corresponding amino acid.

N-substituted derivatives of phenylalanine failed to affect acid production on a phenylalanine-free medium by an organism not requiring this amino acid, suggesting that these compounds do not influence the synthesis of phenylalanine by this organism.

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Magnesium Ion, an Inhibitor of Ribonuclease Activity [1]

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Introduction

The present communication is a report of the inhibition of crystalline ribonuclease activity by quantities of magnesium ion ordinarily serving as activating concentrations for a wide variety of other enzymes. The observation is of particular interest when it is recalled that magnesium has been found to be an activator for the corresponding enzyme depolymerizing desoxyribonucleic acid (1,2).

Method and Materials

The method used was a turbidimetric one described by McCarty (3). At appropriate time intervals 1 ml. of the reaction mixture is removed and mixed with 1 ml. of *N* HCl. The depolymerized nucleic acid is not acid-precipitable so that decrease in turbidity with time is a measure of enzyme activity. The various salt solutions employed were without effect on the turbidity, nor did they cause spontaneous depolymerization of the nucleic acid. This permitted a direct comparison of salt-free systems with test mixtures containing salts. A Coleman Junior spectrophotometer was used to measure the turbidity (transmittance) at 425 mμ.

The turbidimetric method was considered reliable since an inhibitory effect by magnesium ion was also demonstrated by the spectrophotometric method suggested by Kunitz (4). In this procedure the decrease in absorption at 300 mμ which accompanies the digestion of the nucleic acid by enzyme provides the means of measuring activity.

[1] This paper is based upon work done for the Biological Division, Chemical Corps, Camp Detrick, Frederick, Md., under Contract No. W-18-064-CM-227 with the Johns Hopkins University.

Enzyme Solution

A concentrated stock of enzyme was made up in 0.1% sterile gelatin solution and kept in a refrigerator. Before use the enzyme solution was diluted to the desired concentration in a similar gelatin solution. In any turbidimetric experiment 1 part by volume of the diluted enzyme was added to 9 parts of the other reagents so that the concentration of gelatin present in the actual reaction mixture was 0.01%. The presence of gelatin in stock solutions was necessary to prevent inactivation during storage and laboratory manipulation. In a control experiment the effect of magnesium on enzyme activity was found to be independent of the presence of the gelatin. All the data reported involved the use of a crystalline enzyme purchased from Armour and Co., the method of purification being that of McDonald (5). In additional experiments two other preparations of crystalline ribonuclease (one purchased from the Worthington Biochemical Labs. and the other kindly supplied by Dr. R. M. Herriott and prepared by Dr. M. Kunitz) were tested with magnesium and found to be inhibited in activity.

Nucleic Acid

Nucleic acid was dissolved in water as described in (3). Two preparations were used. Prepn. 1 was a commercially prepared yeast nucleic acid purchased from the Nutritional Biochemicals Corp. and used without further purification. A concentration 4 times greater (2 mg./ml.) than that used by McCarty was necessary in order to do experiments by the turbidimetric method. This impure preparation was sufficiently contaminated with extraneous material so that it could not be used with the spectrophotometric method of Kunitz. Prepn. 2 was a commercially prepared yeast nucleic acid from the Bios Labs. which was freed of impurity by a modification of a published method (6).

The study was not limited to the purified material since it was important to learn whether magnesium inhibition of ribonuclease activity was a general phenomenon demonstrable with various preparations of ribose nucleic acid.

Salt Solutions

Analyzed grade dissolved in distilled water.

Results and Discussion

Fig. 1 records the kind of data obtained when $MgSO_4$ was added to a mixture of enzyme and substrate. It is clear that the salt has a notable inhibitory effect on ribonuclease activity, and that the rate of nucleic acid digestion decreases continuously as the reaction proceeds. The minimal inhibitory concentration of salt is less than 0.0005 *M*. An approximately 50% decrease in rate is obtained for each 10-fold increase in molar concentration. That the inhibition was due to the magnesium ion was demonstrated by experiments in which equal molar concentrations of $MgCl_2$ and $MgSO_4$ were directly compared. Each

salt showed the same quantitative inhibitory effects. Thus the inhibition observed is logically attributable to the magnesium. Anions of different valence which accompanied the magnesium would not be expected to have the same quantitative influence.

For those enzymes for which magnesium has been found to act as an activator, it has become customary to test for the effect of additions of fluoride and citrate. In general, the formation of the corresponding magnesium salt or complex has resulted in a reduction of the effect of the magnesium. Therefore experiments were performed to determine

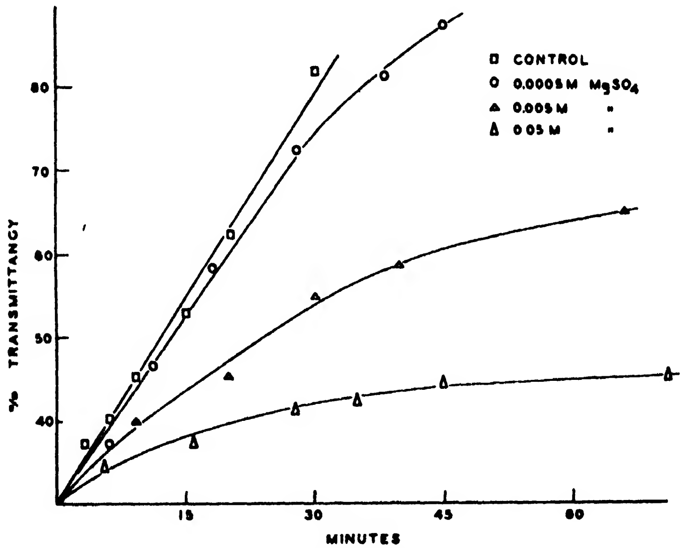

FIG. 1. Effect of varying concentrations of $MgSO_4$ on ribonuclease activity. Reaction mixture at pH 7.2, 34°C. Made up with 2 ml. nucleic acid 1, 0.2 μg. enzyme, 5 ml. 0.025 *M* veronal buffer, 1 ml. $MgSO_4$ solution, distilled water to bring volume to 10 ml.

whether or not the inhibition demonstrated in the present study could be reversed by these substances. Under the conditions used the fluoride and citrate did not give the expected reversal. As a matter of fact, addition of 0.01 *M* NaF enhanced significantly the inhibitory character of magnesium although not inhibitory by itself.

The capacity of low concentrations of magnesium ion to reduce ribonuclease activity was not found to be a property unique for this physiologically important and generally nontoxic cation. Calcium ion acted in the same manner both qualitatively and quantitatively.

Manganese ion also proved inhibitory, but the quantitative data do not permit more than the tentative conclusion that this cation is even more effective than the magnesium. The turbidimetric method for studying the manganese effect is not absolutely reliable since it was found that, unlike the magnesium and calcium salts, mere addition of low concentrations of manganese salts to reaction mixtures caused precipitation of significant amounts of nucleic acid.

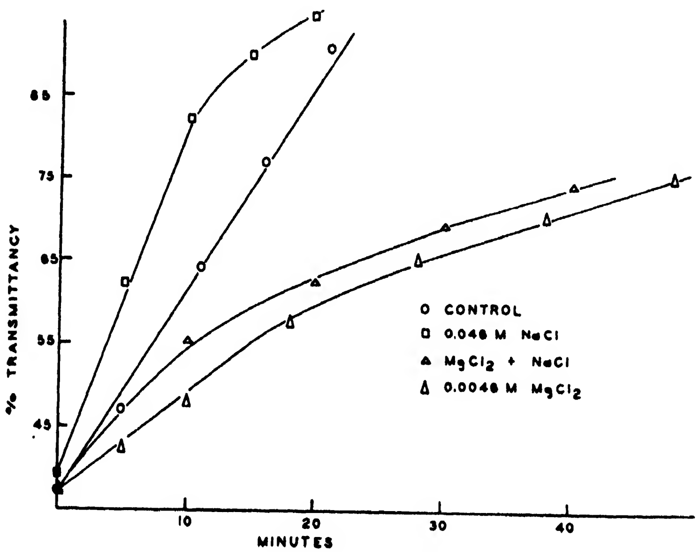

FIG. 2. Effect of NaCl on magnesium inhibition of ribonuclease activity. Reaction mixture at pH 6.8, 34°C. Enzyme: 0.02 μg./ml. veronal buffer: 0.0125 *M*.

Sodium added in the concentration range of 0.1–0.0005 *M* either as the chloride or sulfate stimulated ribonuclease activity, 0.05 *M* being most effective. Higher concentrations had an inhibitory action, 0.33 *M* decreasing the initial velocity approximately 40%. Ammonium as the chloride or sulfate salts was also found to stimulate maximally at about 0.05 *M*. Mixtures of stimulatory concentrations of these salts with magnesium were studied. Inhibition by magnesium was not suppressed. The initial velocities of such mixtures were almost equal to that of the salt-free controls, but the velocity gradually fell to that of the reaction mixtures with magnesium alone (Fig. 2).

The effectiveness of magnesium as an inhibitor was also found to be influenced by the pH of the suspending medium. In Fig. 3 are compared

the inhibitions at pH 7.3 and 8.2. At the latter pH value, which is more nearly optimal for enzyme activity, magnesium had a more pronounced effect in both a relative and absolute sense. The increased inhibition at the more alkaline value is probably directly related to acid-base dissociation rather than to the possibility that activity at any optimal condition is more sensitive to inhibition. This thought is suggested also in observations of the influence of temperature. At 42°C., enzyme activity is greater than at 34°C., the temperature used in all the experiments quoted. Yet inhibition by magnesium proved to be less prominent at 42°C.

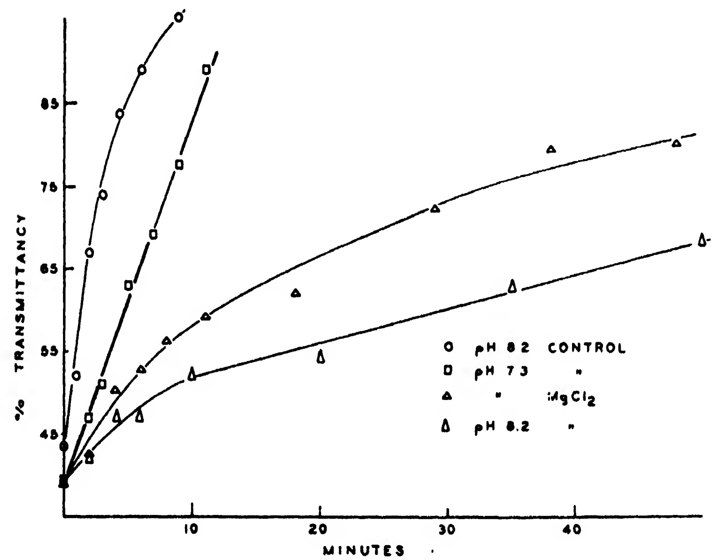

FIG. 3. Effect of pH on magnesium inhibition of ribonuclease activity. Reaction mixture at 34°C. consisted of 2 ml. nucleic acid 1, 0.2 μg. enzyme, 5 ml. 0.02 M veronal buffer, 1 ml. 0.05 M $MgCl_2$, distilled water to bring volume to 10 ml.

For any definitive explanation of the nature and kinetics of inhibition by magnesium it will be essential to know whether the magnesium exerts its influence by combination with the enzyme, substrate, or enzyme-substrate complexes. It would appear that present information as to how magnesium acts as an activator can make little contribution toward suggesting the role of magnesium as an enzyme inhibitor. For though it has been commonly assumed that as an activator magnesium acts directly on the enzyme, this concept has been challenged, and an

effort made to show that the action might actually be on the substrate (7).

In Tables I and II are listed the results obtained with 0.001 *M* $MgCl_2$ when enzyme and nucleic acid concentrations were varied. The data do not indicate any obvious dependence of inhibition on either of the components alone. The data involving nucleic acid as the variable show that enzyme activity may be adversely affected by excess of the substrate itself. Since the effect of substrate concentration on enzyme activity seems not to have been studied by others, this inhibition has not been previously recorded. The effect is particularly pronounced with 2 mg. of purified nucleic acid/ml. of reaction mixture. Furthermore, for the first 30 min. of reaction this system showed a definite lag in attainment of the maximal rate of digestion for both the magnesium-containing test solution and the magnesium-free control. The slopes recorded in Table II for these reaction mixtures were therefore calculated for the maximal activity obtained 30 min. from true zero time. This may logically place an element of doubt on the legitimacy of the comparison of this level of substrate concentration with the other concentrations for which no lag was observed. Since the individual points could not be located with great precision, lines representing the initial slopes (initial velocities) cannot be accurately drawn. Therefore the values of the ratios of the velocities (column 6 of the tables) may not be significantly different. In summation, no obvious explanation of inhibition by magnesium is forthcoming from the experimental data avail-

TABLE I

Effect of Varying Enzyme Concentration on Inhibition of Ribonuclease Activity by Mg^{++}

Nucleic Prepn. 2 employed 1 mg./ml. reaction mixture. Temperature 34°C. Buffer used was 0.0125 *M* veronal. pH values listed are those at end of experiment.

Enzyme concentration µg./ml.	Magnesium chloride				$\frac{\text{Initial velocity control}}{\text{Initial velocity } MgCl_2}$
	Absent		0.001 *M*		
	pH	Slope Initial velocity	pH	Slope Initial velocity	
0.02	8.28	8.6	8.30	4.2	2.0
0.01	8.28	5.3	8.32	3.5	1.5
0.004	8.28	1.5	8.32	0.88	1.7
0.001	8.30	0.40	8.35	0.26	1.5

able, and further studies of the problem must include a definitive analysis of the influence of substrate concentration on ribonuclease activity.

In apparent contradiction to the finding of an inhibitory effect of magnesium on ribonuclease activity is the report of Carter and Greenstein (8) that magnesium, like other electrolytes such as sodium salts, stimulates the enzyme's activity. The method used by these authors for following enzyme activity was not similar to the methods used in the present investigation. They measured the liberation of free-acid groups which accompanies the digestion. Kunitz (6), who originally prepared and studied crystalline ribonuclease, found that the

TABLE II

Effect of Varying Nucleic Acid Concentration (Prepn. 2)

Enzyme concentration 0.01 μg./ml. of reaction mixture. Temperature 34°C. Buffer used was 0.0125 *M* veronal. pH values listed are those at end of experiment.

Nucleic acid concentration	Magensium chloride				Initial velocity control / Initial velocity $MgCl_2$
	Absent		0.001 M		
	pH	Slope Initial velocity	pH	Slope Initial velocity	
mg./ml.					
2.0[a]	8.10	0.76	8.15	0.42	1.8
1.0	8.28	5.3	8.32	3.5	1.5
0.5	8.40	19.	8.47	8.2	2.3

[a] For this concentration of substrate a lag in development of maximal rate of digestion was noted, so that figures quoted for initial velocity are those of maximal activity occurring after a lag of 30 min.

formation of free-acid groups is a slower process than the formation of nonacid precipitable digestion products. The contradiction between the present work and that of Carter and Greenstein (8) may possibly be resolved by considering that reactions were measured which are not directly related. In other words, ribonuclease probably breaks down nucleic acid in successive stages. The first stage, the actual depolymerization, which does not involve the liberation of free-acid groups, is the reaction inhibited by magnesium and is the reaction measured by the turbidimetric and spectrophotometric (4) methods employed in the present studies. Carter and Greenstein, then, have probably measured a

stage of digestion with characteristics apart from those of the first stage.

In view of the fact that magnesium acts as an inhibitor of ribonuclease activity at those concentrations at which it will act as an activator of desoxyribonuclease activity, it is suggested that magnesium may be one of the naturally occurring materials concerned in the regulation of the ratio of ribose and desoxyribose nucleic acids in cells. This function would be exercised by control of the relative rates of breakdown of the nucleic acids concerned.

Summary

Evidence is presented to show that crystalline ribonuclease can be inhibited by concentrations of magnesium ions as low as 0.0005 *M*. No obvious mechanism of the inhibition is suggested by the data at hand.

Independent of the presence of magnesium, a marked rate-reduction of enzyme activity is observed on increasing substrate concentration.

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The Influence of Some Quinone Derivatives on the Lipide Formation in Yeast

Weiss, Fiore and Nord (1) have shown that various naphthoquinones cause a decrease in the fat formation when added to the culture medium of a growing nonpigment producer, *Fusarium lini* Bolley. In this laboratory experiments have been made on the influence of various derivatives of *p*-benzoquinone and the naphthoquinones on the lipide formation in yeast, and it was thought of interest to compare the results of our studies with those obtained in Nord's laboratory.

MATERIAL AND METHODS

A local strain of bottom yeast was used. Ten g. of washed yeast was incubated for 5 or 12 days, respectively, in 100 ml. of a medium which contained 20 g. sucrose, 1.5 g. peptone (Difco), 0.01 g. NaCl, 0.03 g. $MgSO_4$, 0.1 g. KH_2PO_4, and the quinone to be tested, in the appropriate concentration. Then the yeast was centrifuged off, washed twice with 50 ml. of water, and filtered. The total lipide content of the yeast was determined according to Gorbach (2). In an aliquot, the sterol fraction was measured by a method described by Bernhauer (3).

RESULTS AND DISCUSSION

Most quinones (amongst them *p*-benzoquinone, 2,6-dimethoxy-*p*-benzoquinone, 1,2-naphthoquinone, and 1,4-naphthoquinone) did not show any effect on the total lipide content of the yeast, although all these compounds in higher concentrations are powerful inhibitors of yeast growth (4). Three of the quinones tested, however, toluquinone, 4-methoxytoluquinone, and naphthazarin, has a measurable effect on the total lipide content of the yeast. Table I illustrates the action of these three quinones in different concentrations on the total lipide content of the yeast. There was practically no difference in the values obtained after 5 or 12 days of incubation.

It can be concluded from these results, that the sytem converting carbohydrate to fat existing in yeast shows no resemblance to the

TABLE I

Effect of Some Quinones on the Total Lipide Content of Bottom Yeast after 5 Days of Incubation

Figures represent percentage inhibition (−) or activation (+) of lipide formation compared with the controls. Total lipide content in the controls: 31 mg./g. dry weight.

Substance	Molar concentration in the medium		
	10^{-3}	10^{-4}	10^{-5}
Toluquinone	−13	+32	+22
4-Methoxytoluquinone	—	+12	+5
Naphthazarin	—	—	+11

corresponding system in *Fusarium lini* Bolley, as far as sensitivity to quinones is concerned. Though all concentrations in our experiments were appreciably higher than those used by Weiss *et al.* with *Fusarium*, only the highest concentration of toluquinone caused some inhibition of fat formation, whilst in all other cases there was either no effect or an activation took place.

More impressive are the effects on the composition of the total lipides caused by the quinones. With certain concentrations of all quinones tested, we observed a remarkable increase of the sterol portion in the total lipides. The optimal concentration for these effects lies in all cases between 10^{-4} and 10^{-5} *M*. Some typical results of these experiments are collected in Table II.

TABLE II

Effect of Some Quinones on the Sterol Production of Bottom Yeast after 5 Days of Incubation

Figures represent percentage activation compared with the controls. Sterol content in the controls: 4.2 mg./g. dry weight.

Substance	Molar concentration in the medium		
	10^{-3}	10^{-4}	10^{-5}
Benzoquinone	+33	+35	+50
Toluquinone	0	+7	+29
4-Methoxytoluquinone	0	+18	+34
2,6-Dimethoxybenzoquinone	—	+42	+48
1,4-Naphthoquinone	—	—	+12
Naphthazarin	—	—	+35

There obviously exists no correlation between the quinone effects on sterol production and those on the total lipide content of the yeast.

We intend to examine later whether the quinones have an effect on the desaturation of the yeast fats similar to that shown by the naphthoquinone derivative solanione on the fats formed by the nonpigment producer *Fusarium lini* Bolley (5).

A detailed account of our studies will be published later.

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Partition of Tobacco Alkaloids and Some Nicotine Transformation Products on a Paper Sheet Support

The technique of Consden, Gordon and Martin (1) for the separation of amino acids by partition on a filter-paper support has been modified in this laboratory and applied to the resolution of mixtures of nicotine, nornicotine, anabasine, and nine of their derivatives with 15 solvent mixtures. Because the separation is sensitive to pH changes, buffer mixtures have been employed as the water phase. The R_F values for the twelve alkaloids obtained with four of these solvents are presented in Table I.

The positions of the spots were detected by spraying with 1% iodine in 95% ethanol to produce a brown coloration (2). All the possible alkaloid combinations cannot be separated by a single solvent mixture. By selecting appropriate solvents, however, all probable mixtures can eventually be resolved. For futher identification of the alkaloids, we eluted each alkaloid from the paper and obtained its ultraviolet absorption spectrum (3), which was then compared with the absorption curve for the pure material similarly partitioned and eluted.

Using these techniques, we made qualitative and quantitative (3,4) studies of an extract of unfermented cigar-leaf tobacco. This sample, obtained from W. G. Frankenburg of the research laboratory of the General Cigar Company, Inc., Lancaster, Pennsylvania, was an extract of a shed-cured, 1947, Pennsylvania Seedleaf Tobacco (U. S. Type No. 41); it was made as described elsewhere (5) for fraction *A*, and freed of

TABLE I

R_F Values of Alkaloids in Various Solvents[a] at Room Temperature
(Whatman No. 1 Paper[b])

	n-Butanol, 50 Buffer,[c] 50	*n*-Butanol, 85 Benzene 5 Buffer,[c] 30	Methanol, 31 *n*-pentanol, 15 Benzene, 50 Buffer,[c] 8	Butyl acetate, 95 Methanol, 5 0.25% aq. NH_3, 25
N-Methylmyosmine	0.20	0.17	0.26	0.28
2-Hydroxynicotine	0.20	0.18	0.26	0.38
Nornicotine	0.28	0.26	0.32	0.49
Anabasine	0.31	0.32	0.39	0.66
3-(4-Aminobutyl)-pyridine	0.37	0.32	0.30	0.29
Dihydrometanicotine	0.37	0.34	0.35	0.24
Metanicotine	0.40	0.36	0.35	0.41
Nicotine	0.43	0.49	0.80	0.79
Dihydronicotyrine	0.51	0.57	0.85	0.87
Myosmine	0.85	0.87	0.86	0.68
Nornicotyrine	0.91	0.90	0.87	0.82
Nicotyrine	0.91	0.91	0.92	0.85

[a] Composition in milliliters indicated.

[b] The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

[c] The buffer is a mixture of 0.2 *M* acetic acid, 9.5 ml., and 0.2 *M* sodium actate, 90.5 ml.; pH, 5.6.

nicotine by fractional steam distillation in our laboratory. The results indicate that, in addition to nicotine and nornicotine, there are at least three alkaloids but their chemical identities have not been established. One gave an R_F value and an ultraviolet absorption curve identical with those obtained with anabasine. The identities of these alkaloids and details of the technique will be the basis for future papers.

Since the optimum concentration of alkaloid for this technique is

10–50 μg./spot of 1-cm. diameter, or 1–2.5 μg/spot of 2-mm. diameter, if the test tube modification is used (6), a method is now available not only for the isolation and identification of tobacco alkaloids in the plant but also for studying the genesis of alkaloids, their fate during fermentation, their metabolism by animal tissues, and their resolution in pyrolytic products such as in tobacco smoke.

The authors thank C. S. Fenske, Jr., for technical assistance.

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A Relationship of Folic Acid to Choline Oxidase [2]

It has recently been found that during recovery from aminopterin-induced pteroylglutamic acid (PGA) deficiency, monkeys exhibit a marked creatinuria (1). This was interpreted as indicating an effect of PGA on creatine synthesis, and a search was made for specific enzyme systems involved. Choline oxidase seems to be necessary for utilization of the methyl groups of choline for transmethylation processes (2). We have found choline oxidase activity in the livers or kidneys of rats, chicks, rabbits, dogs, and monkeys, the only species so far tested; hence this enzyme may have a rather widespread distribution in the animal kingdom. Choline oxidase determinations were made on livers and kidneys of monkeys injected with aminopterin until death. Measurements of oxygen consumption were carried out by the usual Warburg method. The final volume was 3.0 ml. containing 1 ml. of a 1:3 homoge-

[1] One of the Laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

[2] This investigation was supported by research grants from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

nate of the tissue buffered at pH 7.4. The substrate was 4.0 mg. of choline chloride. Typical results are given in Table I. It may be seen that the liver and kidney of the aminopterin-treated monkey were virtually devoid of choline oxidase activity. We have determined choline oxidase on liver and kidney tissue from 5 aminopterin-treated monkeys and the values were all as small or smaller than those for the aminopterin-treated monkey presented in Table I. We have also determined the choline oxidase activity of liver and kidney tissue from a monkey killed by dietary restriction of PGA, and the values found were quite similar to those obtained from aminopterin-treated monkeys.

TABLE I

Liver and Kidney Enzymes in Control and Aminopterin-Treated Monkeys

Treatment	Tissue	Choline oxidase µl. O_2/g. tissue/hr.[a]	Catalase, mg. H_2O_2 decomposed/g. tissue/min.
Control	Liver	360	44.6
Control	Kidney	430	
Aminopterin	Liver	11	45.1
Aminopterin	Kidney	13	

[a] The difference between tissue + choline and tissue alone.

That this was not merely a nonspecific reduction in enzyme activity is indicated by the fact that catalase activities were quite similar in the livers of normal control and aminopterin-treated monkeys. We have been unable to demonstrate any consistent effect of PGA or aminopterin on choline oxidase *in vitro*.

It is suggested that PGA functions in the choline oxidase enzyme system, possibly as a constituent of the prosthetic group.

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Book Reviews

Biological Antioxidants: Transactions of the Second Conference, October 9–10, 1947, New York, N. Y. Edited by Cosmo G. Mackenzie, Department of Biochemistry, Cornell University Medical College, New York. Josiah Macy, Jr., Foundation, 565 Park Avenue, New York 21, 1948. 116 pp. Price $2.25.

This volume represents the transactions of the Second Conference on Biological Antioxidants sponsored by the Josiah Macy, Jr., Foundation. The 20 participants represent 20 different institutions and industrial laboratories and 12 different subfields of chemical and medical science. The purpose of the several Conferences sponsored by the Foundation is to offer an opportunity for a group of investigators representing, in so far as possible, all the branches of science which bear on a particular medical or health problem to discuss the problem informally. In the words of Dr. Frank Fremont-Smith, Medical Director of the Foundation, the Conferences represent "an experiment in interdisciplinary communication."

In addition to consideration of biological antioxidants in the narrower sense, the group participating has agreed that the control of biological oxidations by naturally occurring inhibitors irrespective of their modes of action also lies within the province of the Conference. With this extension, the scope of the subject discussed offers an obvious opportunity for cross-fertilization of ideas and viewpoints between the medical and chemical sciences concerned. For instance, the tremendous developments in the chemistry of the free radical and the initiation of chain polymerizations by peroxides, the nature of the interaction of molecular oxygen with organic compounds, the puzzling nature of the biological function of the tocopherols, fatty acid chemistry and metabolism, biological oxidations in organized systems and their inhibition, and the mode of action of antioxidants in the organism and in technology, all, accordingly, represent proper subjects for discussion.

Basic to the discussions recorded in these *Transactions* is a series of 9 short, informal resumés of pertinent research and views in some of the scientific areas mentioned.

H. S. Taylor and R. B. Mesrobian present discussions on the physicochemical aspects of chain reactions and their interruption by antioxidants. J. P. Kass discusses polymerization of unsaturated fatty acids from the standpoint of the drying oil technologist. Recent advances in the metabolism of fatty acids are summarized by W. C. Stadie, A. L. Lehninger and W. E. Knox. Inhibition of biological oxidations is considered by Leslie Hellerman. H. P. Morris discusses the results of an investigation on chemical changes occurring in pyrolyzed lard and biological effects produced in rats ingesting such material. Finally, a report on a curious antioxidant effect of butter yellow is given by H. P. Rusch. Considerable discussion, reproduced in the *Transactions*, was aroused by these basic papers.

The papers presented probably have their greatest value as introductory material and are not sufficiently documented to serve as either exhaustive reviews or complete research reports, nor was it the intention that they should be considered as such.

There is evidence in both the prepared papers and the recorded round table discussions of a groping for a common denominator underlying many of the diverse phenomena considered. From the biochemist's standpoint, the most intriguing question concerns the role of free radicals in the mechanisms of intermediary metabolism and biological oxidation. The views of Michaelis concerning one-electron transfers as being basic to biological oxidations are now quite widely accepted. However, some of the cardinal questions are these: Can molecular oxygen cause autoxidation of unsaturated fatty acids *in vivo*? Are such autoxidized fatty acids or their polymerization products injurious to the cell? Is it possible that a fatty acid free radical may react with a free radical of another species formed during the normal course of biological oxidation to form a biologically unwanted or injurious compound, thereby interrupting a biologically essential reaction sequence? To what extent do the tocopherols, as biological antioxidants, serve as controllers or suppressors of such undesirable consequences of the unpaired electron?

These are some of the questions which the reviewer, who was a participant in the conference, mulled over for some time after the conference. The problems discussed may appear to some readers to be too diffuse for a meeting of minds to define, let alone resolve, in a two day period. Perhaps the choice of subject matter formally discussed might have been constricted around a smaller area of the problem with greater profit. In any event, the Conference was an "experiment in communication" and it succeeds or fails, depending on whether it has widened the field of vision of participants or readers of the *Transactions*.

ALBERT L. LEHNINGER, Chicago, Illinois

Studies on Oxygen-Carrying Cobalt Compounds. HARVEY DIEHL AND COWORKERS. A series of Research Papers, reprinted from Iowa State College Journal of Science, Vols. 21 and 22, 1947 and 1948.

The 14 papers reprinted in this volume represent a thorough experimental study of the oxygen-carrying cobalt complexes. The investigation was started for the purpose of oxygen storage. The authors come to the conclusion that the method cannot economically compete with the storage of liquid oxygen. However, the anology with the oxygen-carrying property of hemoglobin makes most interesting material for the biochemist. The problem had been discussed previously in the *Archives of Biochemistry* (**14**, 17 (1947), and **17**, 202 (1949)). The interpretation of the structure differs somewhat from those given by Calvin [*J. Am. Chem. Soc.* **68**, 2254 (1946)], and apparently this work has been executed independently of the latter author. This work is at the same time an interesting contribution to metal-complex chemistry in general. An interesting detail is the fact that not only oxygen but also nitric oxide, but not carbon monoxide, is reversibly bound.

L. MICHAELIS, New York, N. Y.

Annual Review of Microbiology. Vol. 2. 1948. Ed. by CHARLES E. CLIFTON, SIDNEY RAFFEL and H. ALBERT BARKER. Annual Reviews, Inc., Stanford, Calif. 532 pp. Price $6.00.

Those familiar with the *Annual Reviews of Biochemistry* and *of Physiology* may be rather pleasantly surprised at the quality of some of the reviews in this volume. This

statement is not in any way to be taken as derogatory to those two well-established and invaluable publications. It is simply that an annual review, by its very nature, can only chronicle the progress made in a given part of the field during a short period—generally the last year or two—and cannot subject the whole field to critical review and to that synthetic approach which is so difficult to achieve. Because the *Annual Review of Microbiology*, even in this second volume, is covering many fields for the first time, it is not subject to such limitations. It is partly for this reason that the present volume is of exceptional interest.

It is not possible to discuss in detail the 18 separate reviews with their wide coverage, often cutting across several different fields of endeavor and their sometimes enormous bibliographies. Only those which for one reason or another seemed of particular interest to this reviewer will be taken up.

Mrak and Phaff open with a treatment of yeasts which is unusually complete and inclusive. This is certainly one of those "initial reviews" which, as the Editors say in the Preface "will lighten somewhat the task of future reviewers." The references go back to the early thirties and the writers have made good use of their own extensive experience in critically integrating many parts of the scattered field. Their preoccupation with problems of classification and of interrelationships between yeasts and related fungi is somewhat unexpected in these physiological days, and yet is really quite welcome. In other articles one gains the impression that many areas of microbiology are greatly in need of such attention. For instance, in Bailey and Cavallito's valuable discussion of antibiotics (238 references) the general lack of attention to taxonomy on the part of workers in this field is very noticeable. Antibiotics have been prepared from "an unidentified *Actinomyces*," "an *Actinomyces* resembling" certain species, "an as yet unidentified *Actinomyces*," "a species of *Streptomyces*" and "an organism resembling *A. roseus*." In his section on the *Spirochetes*, which again covers the literature of at least 10 years, G. E. Davis gives a detailed discussion and criticism of the classification problem in this group. After describing Bergey's latest proposals, Davis shows how in the one *Spirochaeta* which has been subjected to modern optical and electronic methods of study, its status and that of two of the three genera in the family are called into serious question. He makes clear also that species classification throughout the order is quite confused.

Chemical and biochemical topics, of course, are prominent. Protein Chemistry is represented in two brief reviews, one on "Complement" by Ecker and one on "The Nature of Antibodies" by D. H. Campbell. The latter seemed to be unduly speculative for a review of this type. A remarkably extensive and valuable treatment of neurotropic viruses, by E. W. Schulz, covers four or five years' work in this very active field. All aspects, from the chemistry of virus proteins to clinical and epidemiological work, are included.

The physiological articles include a good discussion of "Bacterial Metabolism" by Gunsalus, which covers only a year of work but still manages to include 177 references. Major advances include the elucidation of the function of two members of the vitamin B group; biotin apparently acts on the formation of oxaloacetate by CO_2 fixation (though Koser in his review of "Growth Factors" brings out a role for biotin also in the synthesis of oleic acid), while pantothenic acid forms coenzyme A for acetylation. "The Physiology of Malarial Parasites," by Moulder, is apparently the first review

ever published on this subject. It seems surprising that, although much of the work has been undertaken with immediate application to war problems in mind, attention should have been directed to the general metabolism of the organisms rather than to the relation between metabolism and growth. Virtanen brings the reader up to date on the current argument over the mechanism of nitrogen fixation. Neither the mediation of hydroxylamine nor the role of leghemoglobin seems yet to be definitely established. A review with the somewhat optimistic title, "The Mode of Action of Chemotherapeutic Agents," is contributed by Hotchkiss; although only covering one year it has nearly 200 references. Despite this volume of work, concrete conclusions, except in the case of the sulfonamides (acting to inhibit the synthesis of pteroylglutamates), seem still to be elusive. Perhaps the most remarkable item is the effect of penicillin on the accumulation of glutamic acid.

A number of misprints, particularly in Latin names, have crept in, but in an undertaking of this magnitude they are probably almost unavoidable. Author and subject indexes are provided, adding considerably to the usefulness of the book. There seems every reason to congratulate both authors and editors on a very valuable contribution to the field.

KENNETH V. THIMANN, Cambridge, Massachusetts

Blood Clotting and Allied Problems. Edited by JOSEPH E. FLYNN. Transactions of the First Conference, February 16–17, 1948, New York, N. Y. Josiah Macy, Jr., Foundation, New York, N. Y. 179 pp. Price $3.25.

Anyone who likes to read the "Congressional Record" will derive much satisfaction from looking through this book which represents an extremely complete transcript of a conference held in February, 1948, under the sponsorship of the Josiah Macy, Jr., Foundation. In many ways this is an amusing anthology, replete with quotations from the Bible (p. 133), from opera (p. 88), and funny poems (p. 101). At a time when polemics have all but disappeared from scientific discussions, it is refreshing to meet a group of scientists who dislike each other's work, and say so, with extreme vigor.

There are few fields in science in which safety valves, such as provided by this conference, are needed as much as in blood coagulation. Had the Tower of Babel been elevated to the rank of a research institute, it could not have included more divergent, mutually exclusive, and often unprovable hypotheses.

The participants in the conference were I. S. Wright, J. E. Flynn, T. Astrup, N. W. Barker, C. H. Best, C. E. Brambel, K. M. Brinkhous, J. T. Edsall, L. B. Jaques, K. P. Link, J. H. Olwin, R. S. Overman, A. J. Quick, W. H. Seegers, H. P. Smith and L. M. Tocantins.

An appendix assembles a comparison of techniques for the determination of prothrombin, as practiced in various laboratories. This was a useful idea.

ERWIN CHARGAFF, New York, N. Y.

Practical Bacteriology, Hematology and Parasitology. By E. R. STITT, PAUL W. CLOUGH and SARA E. BRANHAM. 10th ed., 1948. The Blakiston Co., Philadelphia. xiv + 991 pp. Price $10.00.

The tenth appearance of this handbook, widely used in clinical laboratories, represents an attempt, in general quite successful, to keep abreast of the rapid growth of

the 3 fields which it has sought to embrace. Any book of this kind is impossible to "review" in the best sense of the word, for immediately one is reviewing a compendium of methods of which every worker has a favorite few which he thinks are accurate and reproducible and for which he probably searches.

The section which in such a book is bound to suffer, if only because of space limitations, is that devoted to principles. But it is in this part that space seems not to be the major limitation. The sections on bacterial growth factors express the position in the subject prior to 1936. The utter impossibility of devoting one or two paragraphs to "oxygen requirements" and metabolic products is apparent and only serves to tantalize the reader, for there is no mention of the term oxidation-reduction potential in this section. The sections devoted to the toxic products of bacteria are also couched in vague terms, with no mention of the newer nature of many of the toxins. One could object to the limited directions set forth in the study of many important pathogenic organisms, such as the scant attention paid to media other than Loeffler's for *C. diphtheriae*.

The sections covering medical mycology are well illustrated and organized in a manner well-suited to the laboratorian's needs. The discussion paragraphs dealing with each mycosis are ample for the purpose; but more significant is the care which has been given to the interpretation of laboratory findings. Certain typographical errors are to be expected but some wonder must be occasioned by the consistent use of the term "gramacidin."

Part III of the book deals with Parasitology and, as always in the previous editions of this book, it is a very strong pillar. However, the incorporation in this part of a chapter on poisonous snakes and lizards and one on poisonous fish is difficult to understand. These are trifling points, admittedly, and must not detract from the superbly concise treatment of the protozoan, helminthic, and entomological forms of importance to man. Trophozoites and cysts are described according to their appearance in several suspending media, which is of considerable use.

The section on hematology contains, in addition to the usual descriptions of blood cells and discussions of dyscrasias, a succinct review of the isoagglutination reaction and an extremely compact statement of the Rh antigens. Ample numbers of tables of normal values are available in this chapter.

The treatment of chemical methods of the blood and other body tissues and fluids is complete and quite up-to-date, clearly illustrated, and expressed. Examination of the section on blood alcohol, *etc.*, will provoke "nostalgia" among the alumni of military laboratories who have used this book, especially on late Saturday nights.

SANFORD S. ELBERG, Berkeley, Calif.

Carotinoide. By PAUL KARRER, Director of the Chemical Institute of the University, Zürich, and ERNST JUCKER, Scientific Collaborator at the Chemical Institute of the University, Zürich. Verlag Birkhäuser, Basel, 1948. 388 pp. Price in paper cover, 39 Swiss francs; bound in cloth, 43 Swiss francs.

This is an excellent book, containing a detailed review of our actual knowledge of the chemistry and the biochemistry of the carotenoids; it is a very welcome continuation of the well known monograph by Zechmeister and Cholnoky (1934)

This book consists of a "General Part" (112 pp.) describing the general properties, the distribution and the methods of isolation of the carotenoids, and a "Special Part" (239 pp.) treating in detail of every one of these natural pigments.

The chapter on "Importance of Carotenoids for Plants" (p. 17–19) shows how little is known of this important problem. It seems to the reviewer that Wald's Harvey lecture (1945–1946) contains many interesting facts and hypotheses on the role of carotenoids in phototropism, which could have been mentioned here in detail.

The role of carotenoids as provitamins A and for vision in mammals is treated more fully (p. 19–26). The reviewer wonders whether the vitamins A_1 and A_2, kitol, *etc.*, should not have been included in this book. The fluorescing, colorless C_{40} polyene phytofluene of Zechmeister and coworkers is not mentioned either.

The next 10 pages (27–36) describe in detail the isolation and separation of carotenoids. P. 32 contains a table listing these pigments in the order of their "Position in the Chromatogramm." In view of recent papers of Strain [*Ind. Eng. Chem., Anal. Ed.* **18**, 605 (1946); *J. Am. Chem. Soc.* **70**, 588 (1948)] on the influence of minute changes in solvent composition or adsorbent on the relative position of carotenoids in Tswett adsorption columns, this table has no absolute value.

The chapter on *cis-trans* isomerism of carotenoids (p. 44–49) is a very brief summary of the interesting work of Zechmeister *et al.* Here again, as for instance in the discussion of Wald's excellent work on the retinal cycle, the strong personality of the senior author of the book expresses itself in a very critical manner in reporting the work of other authors. Here and there, some historical points cited by Karrer and Jucker will be objectionable to other authors in the carotenoid field.

Chapter VI (p. 49–58) describes in detail the methods employed for the determination of the constitution of carotenoids and Chapter VII (p. 59–65) the relationship between color and constitution.

Chapter VIII (p. 66–71) is called "Synthesis in the Carotenoid Series," and contains description of the very important work of the authors on epoxides and furanoid carotenoids. It seems to the reviewer that recent synthetic work on vitamin A should also have been mentioned here.

The ninth and last chapter of the general part of the book (p. 71–112) is a very complete compilation of data on the distribution of carotenoids in nature. This chapter has a special list of 434 references (p. 106–112). It took the reviewer a long time to find this list, the location of which is only mentioned in a footnote on p. 72.

The general part contains some useful tables: provitamins A (p. 22), carotenoids classified as epiphasic and hypophasic substances (p. 29), carotenoids classified in order of their absorption bands (p. 64,65).

The "special Part" (p. 113–351) contains a very detailed description of all known carotenoids and their derivatives. The description of the more important pigments is preceded by a short historical introduction and a list of natural sources, with detailed references.

Unfortunately, the authors did not adopt the new "Nomenclature of Carotenoid Pigments" [*Chem. Eng. News* **24**, 1235 (1946)]; the reviewer feels that it would be particularly important to fix the nomenclature of the epoxidic and furanoid carotenoids, for which the suffixes -xanthin, or -chrome are used without distinction.

The natural carotenoids are divided into 5 groups:

1. Hydrocarbons of known constitution (6 pigments, p. 113–171).
2. Hydroxylated carotenoids of known constitution (12 pigments, p. 172–219).
3. Carotenoids of known or partly known structure, containing carbonyl groups (7 pigments, p. 220–260).

4. Carotenoid acids (3 pigments, p. 260–301).
5. Carotenoids of partly or totally unknown structure (37 pigments, p. 302–351).

The major omissions in this last group all concern pigments described by H. H. Strain: the "flavoxanthin-like carotene," or ζ-carotene of carrot roots [*J. Biol. Chem.* **127**, 191 (1939)],[1] the ζ-carotene of the marine diatom *Navicula torquatum* [*J. Am. Chem. Soc.* **65**, 2258 (1943)] neoxanthin of green leaves (Strain, Leaf Xanthophylls, 1938) the four chloroplast pigments of *Dinoflagellatae:* diatoxanthine, diadinoxanthine, dinoxanthine, and neodinoxanthine [*Ann. Revs. Biochem.* **13**, 591 (1944)] and, finally the neo-fucoxanthins A and B from brown algae [Strain and Manning, *J. Am. Chem. Soc.* **64**, 1235 (1942)].

On the other hand, asterinic acid and salmenic acid, of Euler *et al.* have been shown to be identical with astacin and need not be mentioned separately in this list.

The special part is followed by two plates with colored photos of different carotenoid crystals and 13 pages with absorption curves of carotenoids. At the end of the book are two indexes, one for the species of carotenoid-containing plants and animals (p. 367–379) the second, a general index, in which we have missed some entries (*e. g.*, rubichrome, citroxanthine).

There are very few printing errors; the reviewer has only found one of importance: p. 336 the formula of pectenoxanthine is $C_{40}H_{54}O_3$ (and not $C_{40}H_{54}O_2$).

The book is very well printed and solidly bound and is a very welcome and important addition to modern biochemical literature.

E. LEDERER, Paris, France

The Cytoplasm of the Plant Cell. By ALEXANDRE GUILLIERMOND. Chronica Botanica Co., Waltham, Massachusetts, 1948. x + 247 pp. Price $5.00.

This book should attract the attention of a large number of scientific readers interested in the foundational structure of living matter.

Today, as we are realizing the significance of microstructure and the prominence that structure is occupying in physiological and biochemical processes, we can claim that a situation of considerable satisfaction to the morphologist has arrived. Improved optical methods are revealing a variety of microstructures. We are now in need of a good summary of the microscopically visible cytological structures so that we may relate them, wherever possible, with ultra structures such as are becoming evident, for example, with the electron microscope. The morphological cytologist has always claimed that structures consistently revealed by fixation or by direct observation of of living cells are in all likelihood composed basically of similar structures *in parvo*.

This phase of Guilliermond's work is brought out in the book under review. An appreciation of it can best be attained by the realization that it is founded on years of close attention to morphological and developmental studies chiefly among the ancestral types of the vegetable kingdom.

The author, whose death occurred in 1947 at the age of 71, was chairman of the Department of Botany at the Sorbonne, Paris. His numerous researches began with extended taxonomic and cytological studies of yeasts and other lower Fungi with a view of investigating the phenomenon of sexuality among the *Thallophytes*. These studies in tracing the origin and phylogeny of the yeasts and their relations with

[1] See also Nash and Zscheile, *Arch. Bioch.* **7**, 305 (1945).

bacteria, led to controversial fields which engaged much of his time throughout his life. His identification of the metachromatic corpuscles of Babe's in bacteria with similar intracellular structures in the yeasts led him to the search of similarly-staining granules throughout the lower orders of the vegetable kingdom, and to the concept of their general existence as reserve products.

These intensive researches on the finer details of yeast cytology developed into investigations of the morphological constitution of the vegetable cytoplasm and its relation to the formed constituents which were becoming recognized in the cytoplasm of animal cells. Guilliermond's greatest contribution with that of his students arose from his painstaking, detailed microscopic studies of the genesis of cytoplasmic organellae, in particular of the mitochondria and of cell vacuoles.

He established the existence in the yeast cell of a definitely structured nucleus independent of the metachromatic corpuscles which hitherto had been regarded as representing a diffuse "nucleus." During the budding process in Saccharomyces the nucleus may divide amitotically. During sporulation of a closely related form, the *Schizosaccharomyces*, the division is mitotic.

His later work dealt with cytoplasmic structures in the entire vegetable kingdom, comparison being made with cytoplasmic structures in animal cells. Great credit is due him for his continued emphasis on living cells, in which his studies were directed toward comparing embryonic with adult cells with regard to the progressive evolution of the cytoplasmic structures.

The first 5 chapters of the book are introductory in nature and present, in an elementary way, generally accepted views of the physical properties and the chemical and physicochemical constituents of the cytoplasm. The rest of the book deals with the special province of the author's researches. Six chapters are devoted to the chondriome, a system of cytoplasmic structures which include mitochondria (granulae), chondrioconts (filamentous), and also the plastids. Five chapters cover the vacuolar system and its vital staining properties. The last 3 chapters deal with incidental structures which are not consistently found in the cytoplasm, or which may appear in dying cells. It is significant that the evidence for the Golgi apparatus, which has been widely described in animal cells, are treated by the author as being of dubious value.

The author, as quoted from his summary and conclusions has the following to say concerning the 2 chief systems present in the cytoplasm of plant cells:

"The chondriome: This is composed of elements which are elements in the form of granules (mitochondria), of short rods, or of filaments which may be grouped or branched (chondrioconts, 0.5–1 thick). They are capable of dividing and of passing from one to the other of these forms, either by elongations of the mitochondria or by fragmentation of the chondrioconts. The mitochondrial form is generally the one which characterizes the sexual cells and the early stages of embryonic cells. The chondriocontal form is generally the one which is the most widespread throughout differentiated cells.

"The chondriosomes are visible in living material in which they appear as slightly refractive elements whose forms are exactly like those obtained in fixed preparations. From a chemical point of view, the chondriosomes have a lipoprotein constitution and seem to be composed of a protein and phosphoaminolipide complex which is much richer in lipides than is the cytoplasm. The chemical behavior of the chondriosomes is absolutely different from that of chromatin and they do not show the Feulgen reaction.

"There has been attributed to chondriosomes in animal cells a preponderant and direct role in the elaboration of metabolic products; fats, granules resulting from all sorts of secretion, pigments. According to this concept, the chondriosomes have a role rather analogous to that of plastids in chlorophyll-containing plants.

"The plastids: The cells of chlorophyll-containing plants are distinguished from the cells of animals and fungi by the presence of a second category of organellae, the plastids. It is definitively proved that these organellae form only by division of preexisting plastids and which maintain their individuality in the course of development.

"The plastids may be considered as a supplementary category of chondriosomes connected with the photosynthetic function which characterizes green plants. Hence, we are led to think that the ordinary chondriosomes and the plastids, by virtue of their finely divided state in the cytoplasm, are the seat of important surface phenomena and that they have a similar general function, of which that, shown by the plastids, is one manifestation.

"Vacuolar system or vacuome: This is represented in embryonic cells of most plants by numerous minute inclusions of semifluid consistency composed of a very concentrated colloidal solution (in the state of a hydrogel or coacervate). In their forms (granules, isolated or assembled in chains, undulating filaments often anastomosing into a network), they sometimes greatly resemble the chondriosomes. These inclusions are occasionally visible in living material because they are more refractive than the cytoplasm, or because they contain anthocyanin pigments which give them a natural color. They are most difficult to distinguish under the ultramicroscope, for here they look like chondriosomes. They can be easily brought out with vital stains (neutral red, cresyl blue, Nile blue), for which they have a strong predilection . . . In the course of cellular differentiation, these elements swell because they contain colloids in pseudosolution whose capacity for taking in water is much greater than that of the cytoplasm. Thus, they are transformed into small, spherical, increasingly fluid vacuoles (vacuoles in the classical sense). There has taken place, therefore, a transformation of the very condensed colloidal substance, of which they seem to have been formed, into a very dilute solution. The vacuoles may later fuse so that, in the mature cells, there is formed a single enormous vacuole.

"The large vacuole of mature cells is capable, under certain influences, of losing its water and of fragmenting into minute, semifluid, chondriosome-shaped elements. The various aspects of the vacuolar system are, therefore, reversible and seem to depend upon water content of the cytoplasm. Water may move into the cytoplasm and out of the vacuoles and the reverse action may take place.

"Although in their semifluid state the vacuoles may very much resemble the chondriosomes and the plastids, they are always distinguishable from these elements by their histochemical behavior, notably by their instantaneous staining with vital dyes such as neutral red and cresyl blue, which stain neither the chondriosomes nor the plastids. They are to be distinguished from these elements also by the fact that the staining is essentially vital and ceases as soon as death of the cells occurs, whereupon the protoplasm is stained. This is very different from the sublethal staining of the chondriome which almost never occurs, except in dying cells, and persists after the death of the cells.

"Although almost always represented in plant cells, and capable of fragmenting, the vacuoles seem to arise *de novo*. It may be supposed that their formation is, in general, connected with secretory phenomena of the cell. Each colloidal granule

secreted by the cytoplasm, possessing a capacity for taking in water which is greater than that of the cytoplasm, seems capable of engendering a vacuole."

This book constitutes Volume 6 of Frans Verdoorn's "New Series of Plant Science Books" published by Chronica Botanica Co (Waltham, Mass.) of which Dr. Verdoorn is Managing Editor. The book, written in French, was translated by Dr. Lenette Rogers Atkinson, a former C. R. B. Fellow in Botany (University of Louvain). It has not been published in French. The price of the book is $5.00 and may be obtained either from the Chronica Botanica Co., Waltham, Mass, or from Stechert-Hafner, Inc., New York City. The book was first published in 1941 and was reprinted in 1948.

ROBERT CHAMBERS, New York, New York

Annual Review of Biochemistry. Editor, J. MURRAY LUCK. Annual Reviews, Inc., Stanford, Calif., 1948. vi + 801 pp. Price $6.00.

The general style, and the persistently high standard, of the *Annual Review* may be assumed to be well known to every reader of this journal, and only a few points need special mention on the occasion of the appearance of the new volume. Apart from the chapters dealing with the regular subjects, Vol. XVII contains sections on The chemistry of the Immunopolysaccharides (W. N. Haworth and M. Stacey), X-Ray Crystallographic Studies of Compounds of Biochemical Interest (D. Crowfoot), The Biochemistry of Carcinogenesis (H. P. Rusch and G. A. LePage), The Terpenes (in Relation to the Biology of *Genus Pinus*) (N. T. Mirov), The Chemistry of Penicillin (E. Chain), Ruminant Digestion (S. R. Elsden and A. T. Phillipson), and Physiological Aspects of Genetics (G. W. Beadle).

In the preface the editors reflect on the growth of scientific publications. They quote A. W. Hulme for the statement that the yearly average of scientific papers in the pure sciences was close to 3100 for the period 1800 to 1863, rising gradually to about 85,500 in 1910. The rate of publication in 1934 has been estimated at 750,000 "good" or "fair" papers per year.

The more recent growth of biochemistry has confronted the editors with a decision between two alternatives, neither of which they regarded as being without disadvantages. Either the allocation of space could be made even more rigorous and severe, or a portion of the subject matter, "rather clearly contained in a few of the chapters," could be transferred to a new review. The latter alternative was chosen and it is proposed to publish, form 1950 onwards, an Annual Review of Plant Physiology. Few will quarrel with this decision.

After studying a number of chapters in detail the reviewer has no major criticisms to offer. The present volume, as its predecessor, is an invaluable mine of information to all workers in the field of biochemistry.

H. A. KREBS, Sheffield, England

Erratum

Volume 23, Number 2, p. 323

Line 8 of Table I should read, "As 3 + 7-dehydrocholesteryl bromide," instead of, "As 2 + 7-dehydrocholesteryl bromide."

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